Interaction of REPS2 with NF-κB in Prostate Cancer Cells

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Interactie van REPS2 met NF-κB in prostaatkankercellen

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List of Abbreviations

AD	activation domain
ADPC	androgen-dependent prostate cancer
AIPC	androgen-independent prostate cancer
AML	acute myelogenous leukaemia
ANK	ankyrin
AP2	adapter-related protein complex 2
AP2A	adaptor-related protein complex 2, alpha subunit
AR	androgen receptor
ARF	ADP-ribosylation factor
ARFGAP	ADP-ribosylation factor GTPase activating protein
BCL2	B cell CLL/lymphoma 2
CDC42	cell division cycle 42
CHO-IR	Chinese hamster ovary cells that express human insulin receptor
CHUK	conserved helix-loop-helix ubiquitous kinase (formerly known as IKK1 and IKK-alpha)
COS cells	cell line derived from monkey kidney cells
DBD	DNA-binding domain
DDEF1	development and differentiation enhancing factor 1
DHT	dihydrotestosterone
EGF	epidermal growth factor
EGFR	EGF receptor
EH	Eps15 homology
EPN1	epsin 1
EPS15	epidermal growth factor receptor pathway substrate 15
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog [avian]
EVH1	ena-VASP homology 1 domain
FW	phenylalanine-tryptophan
GEF	guanine nucleotide-exchange factor
GRB2	growth factor receptor-bound protein 2
H(S/T)F	histidine-serine/threonine-–phenylalanine
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
IGF1	insulin-like growth factor 1 (IGF1)
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta (formerly known as IKK2 and IKK-beta)
IKBKG	inhibitor of kappa light polypeptide gene enhancer in B cells, kinase gamma (formerly known as NEMO)
IKK	IκB kinase
IL1	interleukin 1
IL4	interleukin 4
IPT	immunoglobulin-like fold, plexins, transcription factors
JC19	code name, later renamed to REPS2
KGF	keratinocyte growth factor
LNCaP	lymph node carcinoma of the prostate
LNCaP-FGC	LNCaP fast-growing colony
LNCaP-LNO	LNCaP lymph node original
MAP	mitogen-activated protein

MPCs	membrane-permeable compounds
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA1	nuclear receptor coactivator 1
NCOA2	nuclear receptor coactivator 2
NF-κB	nuclear factor κ chain transcription in B cells
NLS	nuclear localisation signal
NPF	asparagine-proline-phenylalanine
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PMA	phorbol 12-myristate 13-acetate
POB1	partner of RALBP1
PRM	proline rich motif
PTM	post-translational modification
RAC1	ras-related C3 botulinum toxin substrate 1
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RAL	v-ral simian leukemia viral oncogene homolog
RALA	v-ral simian leukemia viral oncogene homolog A
RALBP1	RALA binding protein 1
RALGAP	Ral GTPase activating protein
RALGDS	ral guanine nucleotide dissociation stimulator
RAS	rat sarcoma viral oncogene homolog
REPS2	RALBP1 associated Eps domain containing 2
RHD	Rel homology domain
RHO	Ras homolog gene family
RHOA	Ras homolog gene family, member A
RHO-GAP	RHO-GTPase-activating protein
RING	really interesting new gene
RME	receptor-mediated endocytosis
RTK	receptor tyrosine kinase
SH2	Sarc homology 2
SH3	Sarc homology 3
SOS	son of sevenless
STAT6	signal transducer and activator of transcription 6
SWG	serine—tryptophan-glycine
T	testosterone
TAD	trans-activation domain
TNF	tumor necrosis factor
TRAF4	TNF receptor-associated factor 4
WW	tryptophan-tryptophan
WW domain	protein-protein interaction module composed of 35-40 amino acids, with two conserved tryptophans (W) spaced 20-22 amino acid residues apart
zf-TRAF	TRAF-type zinc finger

CHAPTER 1

General Introduction

General Introduction

1.1 Prostate cancer

Prostate cancer, one of the most diagnosed and deadly cancers in the western world, passes through different stages. In the first two stages - according to the tumour, node, and metastasis (TNM) classification (Schroder et al., 1992) - prostate cancer cells are located only in the prostate gland, whereas in the third stage the cancer cells have spread outside the covering (capsule) of the prostate gland into tissues around the prostate but not to the lymph nodes. In the fourth stage, the cancer cells have spread (metastasised) to lymph nodes near or far from the prostate gland, or to organs and tissues far away from the prostate, such as the bone, liver and lungs. Like normal prostate cells, prostate cancer cells are dependent on androgen receptor (AR) signalling, induced by androgens, for growth and survival. The developing and fully developed normal prostate requires continuous exposure to androgens, and loss of androgenic stimulation leads to massive apoptosis of prostate cells (Denmeade et al., 1996). Likewise, without androgens present, androgen-dependent prostate cancer (ADPC) cells will die (Denmeade et al., 1996; Huggins, 1967). Because of this, ADPC can be treated by ablation of androgens through surgical or chemical castration. However, despite the deprivation of androgens, inevitably some of the prostate cancer cells will manage to survive and continue to grow at a very low level of androgen exposure or even independent of androgens. This development of androgen-independent prostate cancer (AIPC) during hormone deprivation or antihormonal treatment is a serious health problem, since AIPC cannot be treated effectively.
Many studies have focussed on molecular mechanisms that may underlie survival of prostate cancer cells during androgen deprivation. These studies demonstrate different molecular mechanisms that may lead to AIPC and contribute to the ability of prostate cancer cells to survive androgen ablation therapy. Some of the identified mechanisms are briefly described below.

1.2 AR dependent advanced prostate cancer

Amplification of the *AR* gene or AR protein stabilization

Amplification of the wild-type *AR* gene has been found in tumours from patients with recurrent prostate cancer (Koivisto et al., 1997; Visakorpi et al., 1995). Approximately 30% of the recurrent therapy-resistant tumours, but none of the untreated primary tumours, were found to contain *AR* gene amplification. This amplification was associated with a substantially increased level of mRNA expression (Koivisto et al., 1997), which probably makes the cells capable to attain an active androgen receptor pathway also at a low level of androgens (Koivisto et al., 1997). Another report describes that the AR protein is more stable in recurrent prostate cancer cells, compared to the AR in androgen-dependent cells (Gregory et al., 2001b).

This increased stability of the AR was associated with an increased sensitivity of the cells to a growth-promoting effect of the androgen dihydrotestosterone (the two most important androgens are testosterone and its metabolite dihydrotestosterone) (Gregory et al., 2001b). The concentration of dihydrotestosterone required for growth stimulation in recurrent prostate cancer CWR-R1 and LNCaP-C4-2 cells is four orders of magnitude lower than that required for androgen-dependent LNCaP cells (Gregory et al., 2001b).
In many cases, cells with amplification of the *AR* gene or with a more stable AR protein still need exposure to androgens, at a low level, to survive and grow. Still, such cells are usually considered as AIPC, which implies that the name AIPC cells does not apply only to cells that have become completely androgen independent.

Mutations in the AR

The wild-type AR has a high and hormone-specific affinity for the androgens testosterone (T) and dihydrotestosterone (DHT), which bind to the ligand-binding domain, the first step in activation of the androgen receptor pathway. However, it has been reported that mutations in the AR can modify this specificity, such that the mutated AR can be activated not only by androgens but also by other ligands (Steketee et al., 2002; Veldscholte et al., 1990). Selective pressure on prostate cancer cells during androgen ablation therapy might increase the survival of cells with mutations in the AR. In particular if mutations would result in activation of the AR by other ligands such as anti-androgens used in therapy, androgen ablation therapy of prostate cancer patients might become ineffective. Also, a mutated AR may bind other ligands that are normally present in the human body. For example, in prostate cancer cells from a patient who failed to respond to androgen ablation therapy, a double-mutated AR has been detected, and this double-mutated AR was found to have a high binding affinity for glucocorticoids (Brinkmann & Trapman, 2000; Zhao et al., 2000). The physiological concentration of glucocorticoids in men is high enough to activate this double-mutated AR.

Ligand-independent activation of AR

Activation of endogenous wild-type AR induced by growth factors, may be another mechanism for androgen-independent growth of prostate cancer cells during androgen ablation therapy. For example, Culig et al. (1994) demonstrated AR mediated activation of an androgen-responsive reporter by exposure of cells to insulin-like growth factor I (IGF1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF). Most likely, the mechanisms involved activate the AR in an indirect manner, following activation of growth factor signalling pathways. Several growth factors are over-expressed in some prostate cancers. However, Culig et al. (1994) also observed that activation of the AR pathway by IGF1, KGF and EGF was completely blocked by the AR antagonist Casodex. Hence, it is not likely that activation of the AR by growth factor signalling is responsible for, or contributes to, AIPC in patients who fail to respond to Casodex therapy.
The ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; also known as HER-2/neu) receptor tyrosine kinase is also able to activate the AR pathway in the absence of androgens (Craft et al., 1999). Furthermore it was demonstrated that ERBB2 could synergy with a low level of androgen to ‘superactivate’ the AR pathway. Because it was found that ERBB2 is expressed at a relatively high level in

AIPC cells compared to ADPC cells, over-expression of ERBB2 might be another mechanism through which AIPC cells can escape cell death under conditions of androgen ablation (Craft et al., 1999).

Increased or decreased expression of AR coregulators

Nuclear receptor coregulators are coactivators or corepressors that are required by nuclear receptors for efficient transcriptional regulation (McKenna et al., 1999). The transcriptional activity of the AR can be affected by coregulators, since coregulators can influence a number of functional properties of the AR, including ligand selectivity and DNA binding capacity (Heinlein & Chang, 2002). Aberrant coregulator activity due to mutation or altered expression levels may cause activation of AR also if androgen levels are low, and therefore contribute to the progression of prostate cancer.
Gregory et al. (2001a) have reported that a majority of recurrent prostate cancers express high levels of the androgen receptor and two nuclear receptor coactivators, namely nuclear receptor coactivator 1 (NCOA1, also known as SRC1) and nuclear receptor coactivator 2 (NCOA2, also known as TIF2). Overexpression of these coactivators increased AR transactivation at a physiological low concentration of adrenal androgens (Gregory et al., 2001a). So, through overexpression of AR, NCOA1, and NCOA2, prostate cancer cells might become less responsive to androgen ablation therapy and escape cell death.

1.3 AR independent advanced prostate cancer

Androgen ablation therapy initially results in lack of activation of the AR pathway, causing regression of ADPC tumours. However, at low levels of androgens during androgen ablation therapy, some reactivation of AR signalling may occur (see the described mechanisms above), which can contribute to survival of prostate cancer cells during therapy. Besides this aberrant reactivation of AR signalling, other pathways might play a role in survival of prostate cancer cells during androgen ablation, for example pathways that enhance cell survival or inhibit apoptosis.

BCL2

A protein that might play a role in survival of prostate cancer cells independent of the AR, is BCL2 (B cell CLL/lymphoma 2), which can suppress apoptosis. It has been reported that BCL2 expression is increased in AIPC cells compared to ADPC cells, and that this increase might result from androgen ablation (Colombel et al., 1993; Liu et al., 1996; McDonnell et al., 1992). Additionally, adjuvant use, combined with androgen ablation, of antisense BCL2 oligodeoxynucleotide, which downregulates BCL2 expression, was found to delay progression to androgen independence in a LNCaP prostate tumour model in mice (Gleave et al., 1999). Although the apoptosis suppressor BCL2 seems to play an important role in prostate cancer progression, *BCL2* gene expression is not essential for either androgen independence or metastatic ability of human prostatic cancer cells (Furuya et al., 1996). Prostate cancer cells may survive cell death signals induced by androgen ablation through a mechanism that involves BCL2, but also through other mechanisms.

MYC

Another protein that can play an important role in AIPC development independent of the AR is MYC (v-myc myelocytomatosis viral oncogene homolog, also known as c-Myc). Amplification of the MYC proto-oncogene is a frequent alteration in hormone refractory prostate carcinomas (HRPC). Bernard et al. (2003) showed that human androgen-dependent prostate cancer cells overexpressing MYC grew independently of androgens and presented tumorigenic properties in androgen-depleted conditions. Moreover, RNA interference directed against MYC showed that growth of human AIPC cells, AR-positive or -negative, required MYC expression (Bernard et al., 2003). Transgenic mice expressing human MYC in the mouse prostate developed murine prostatic intraepithelial neoplasia followed by invasive adenocarcinoma (Ellwood-Yen et al., 2003). Targeted inhibition of MYC expression using a specific morpholine antisense oligomer caused significant growth inhibition and apoptosis in prostate cancer cells and in xenografts (Iversen et al., 2003). Phase I safety trials of this morpholino in humans showed no toxicity or serious adverse events. Therefore inhibition of MYC expression by antisense phosphorodiamidate morpholino oligomer is a promising new therapeutic strategy for prostate cancer (Iversen et al., 2003).

ID1

ID proteins contain a helix-loop-helix (HLH) motif and regulate tissue-specific transcription within several cell lineages. They do not bind DNA directly, but inhibit lineage commitment by binding basic helix-loop-helix (bHLH) transcription factors through their HLH motif. ID proteins contribute to cell growth, senescence, differentiation, and angiogenesis. The ID1 (inhibitor of DNA binding 1) protein is a dominant-negative regulator of basic helix-loop-helix transcription factors. ID1 controls malignant cell behaviour in many different tissues. Prostate cancer progression is accompanied with increased ID1 expression (Ouyang et al., 2002). The ID1 protein was up-regulated during human prostate cancer progression in vivo and was overexpressed in highly aggressive prostate cancer cells (Coppe et al., 2004; Ling et al., 2004). ID1 may serve as a molecular marker of aggressive human prostate cancer (Coppe et al., 2004). In prostate cancer cells, ID1 promotes cell survival through activation of the NF-κB signalling pathway (Ling et al., 2003). Interestingly, inactivation of ID1 by its antisense oligonucleotide and retroviral construct in DU145 (which is an AIPC cell line that has a constitutively active NF-κB pathway; see also below) cells resulted in a decrease in NF-κB activity and this was associated with increased sensitivity to TNFα-induced apoptosis (Ling et al., 2003). On the other hand, constitutive expression of ID1 converted nonaggressive LNCaP prostate cancer cells into more proliferative and invasive cells and increased their secretion of matrix metalloproteinases (Coppe et al., 2004). Inactivation of ID1 may lead to sensitization of prostate cancer cells to chemotherapeutic drug-induced apoptosis, and may therefore be a potential therapeutic target (Ling et al., 2004; Ling et al., 2003). ID1-induced androgen-independent prostate cancer cell growth was correlated with up-regulation of EGF-R (epidermal growth factor-receptor) and PSA (prostate specific antigen) expression (Ling et al., 2004). In contrast, down-regulation of ID1 in androgen-independent DU145 cells by antisense oligonucleotides resulted in suppression of EGF-R expression at both transcriptional and protein levels (Ling et al., 2004).Taken together, up-regulation of ID1 in prostate cancer cells may be one of

the mechanisms responsible for developing androgen independence and this process may be regulated through induction of EGF-R expression.

RAS mediated growth factor signalling

Survival of prostate cancer cells after androgen ablation therapy is associated with upregulation of growth factor (GF) mediated pathways (Russell et al., 1998; Ware, 1998). Many of these growth factor pathways utilize the small G protein RAS as part of their normal signalling activities (see also Figure 2). Bakin et al. (2003b) showed that stable expression of RAS effector-loop mutants that activate the RAS/MAP kinase pathway is sufficient to reduce the androgen requirement of LNCaP prostate cancer cells for growth, prostate-specific antigen expression, and tumorigenicity. In another study, Bakin et al. (2003a) showed that decrease of RAS signalling restores androgen sensitivity in hormone-refractory C4-2 prostate cancer cells.

NF-κB

The transcription factor nuclear factor-kappaB (NF-κB) promotes the production of angiogenic, antiapoptotic, and prometastatic factors that are involved in carcinogenesis. The NF-κB pathway may play a role in the progression of prostate cancer since this pathway is inactive in ADPC, but constitutively active in AIPC (Chen & Sawyers, 2002; Gasparian et al., 2002; Palayoor et al., 1999). Through this constitutively active NF-κB pathway, AIPC cells express specific proteins that promote cell survival. For example, the *BCL2* gene (see also above in this Paragraph) is transcriptionally regulated by NF-κB (Catz & Johnson, 2001). PSA (prostate-specific antigen), an important marker for androgen-independent prostate cancer, is also transcriptionally regulated by NF-κB (Chen & Sawyers, 2002). Lindholm et al. (2000) demonstrated that increased NF-κB activity contributed directly to the invasive behavior of PC-3 prostate cancer cells. On the other hand, Huang et al. (2001) showed that blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Inhibition of the NF-κB pathway in prostate cancer cells is and has been the focus of many studies. Several natural substances seem to have an inhibiting effect on the NF-κB pathway in prostate cancer cells, including Genistein, an isoflavone from soya bean (Davis et al., 1999), grape seed extract (Dhanalakshmi et al., 2003), zinc (Uzzo et al., 2002), and apigenin, which is a common plant flavonoid (Shukla & Gupta, 2004). These substances may be interesting for targeting NF-κB for the prevention and/or treatment of prostate cancer.

1.4 REPS2 (JC19/POB1) is downregulated in AIPC

In the above Paragraphs (1.2 and 1.3), several mechanisms are described that may play a role in the transition from ADPC to AIPC (Paragraph 1.1). Not all the mechanisms that have been identified so far were described here, and undoubtedly there are unidentified mechanisms through which prostate cancer cells can counteract cell death signals induced by androgen ablation. By finding differentially expressed genes between ADPC and AIPC, extended knowledge on these mechanisms and/or new mechanisms can be identified.

JC19 is downregulated in androgen-independent prostate cancer

To find differentially expressed genes between ADPC and AIPC, our laboratory conducted a differential display study (Chang et al., 1997). Differential gene expression between androgen-dependent (LNCaP-FGC) and androgen-independent (LNCaP-LNO) prostate cancer cells were investigated using RNA arbitrarily primed, and differential display PCR of mRNA (Chang et al., 1997). This resulted in identification of four cDNAs that are differentially expressed between LNCaP-FGC and LNCaP-LNO, and one of these transcripts was code named JC19. The *JC19* gene was higher expressed in LNCaP-FGC cells compared to LNCaP-LNO cells. Although JC19 is differentially expressed between androgen-dependent and androgen-independent prostate cancer cell lines, it is not androgen regulated (Chang et al., 1997). Expression of the JC19 mRNA was subsequently studied using a panel of human prostate cancer xenografts, which are human prostate cancer tissues that are growing under the skin of mice (van Weerden et al., 1996). In androgen-dependent xenografts, the JC19 mRNA level was much higher compared to its expression in androgen-independent xenografts, in which expression was hardly detectable. The mRNA expression pattern in the xenografts was in good agreement with that observed in the LNCaP-FGC and LNCaP-LNO cell lines (Chang et al., 1997). In addition, differential expression of JC19 has been observed in patient tumour samples (unpublished data). Interestingly, in several estrogen-independent breast cancer cell lines JC19 expression was also virtually lost as compared to in estrogen-dependent breast cancer lines (unpublished data). Taken together, JC19 seemed to be an interesting protein for further examination. JC19 cDNA was cloned and appeared to contain an open reading frame for a protein of 659 amino acid residues. The JC19 sequence was submitted to and named by GenBank to REPS2 (RALBP1 associated Eps domain containing 2).

Protein-protein interaction regions in REPS2

The 659 amino acid sequence of the REPS2 protein has some regions that are possibly involved in protein-protein interaction. In order from the N-terminal to the C-terminal side, REPS2 has: a proline-rich motif (PRM), an EPS15 Homology (EH) domain, three proline-rich motifs, and a coiled-coil domain (Figure 1).
The EH domain is a protein-binding domain that was first identified in the receptor tyrosine kinase substrate EPS15, and is conserved in evolution (Wong et al., 1995). EH domains are ~100 amino acids long, and proteins that contain one or more of these domains may be involved in endocytosis, actin remodelling, and intracellular transduction of signals. Phage display experiments identified three classes of peptides that are able to bind the EH domain (Paoluzi et al., 1998). The majority of EH domains bind preferentially to peptides containing an asparagine-proline-phenylalanine (NPF) motif (class I peptides). However, binding to peptides containing phenylalanine-tryptophan (FW), tryptophan-tryptophan (WW) or serine-tryptophan-glycine (SWG) motifs (class II peptides) was also found. A third class of ligands, containing a histidine-serine/threonine-phenylalanine (H(S/T)F) motif, was found to bind exclusively the first EH domain of End3p, a protein that is involved in endocytosis in yeast (Confalonieri & Di Fiore, 2002; Paoluzi et al., 1998). In addition, using a modified Far Western technique, direct screening of a cDNA expression library with EH domains yielded a number of putative EH interactors, all of which possessed NPF motifs that were shown to be responsible for the interaction (Salcini et

al., 1997). These putative EH interactors displayed no sequence homology among themselves, except for the presence of one or more copies of the NPF motif.
It is generally thought that proline-rich motifs bind to proteins that contain SH3, WW, or EVH1 domains (Kay et al., 2000). The core motif that binds to the SH3 domain is P*XX*P, and this motif is further classified into class I and class II. The class I motif is (R/K)*XX*P*XX*P and the class II motif is P*XX*P*X*(R/K). C-terminal from the EH domain, REPS2 has three proline-rich motifs (Figure 1). All of these proline-rich motifs represent class II SH3 domain-binding motifs. Further, on the N-terminal side of the EH domain, REPS2 has a proline-rich motif representing a class I WW domain-binding motif (Figure 1).
On the most C-terminal side of REPS2, a coiled-coil structure motif is located (Figure 1). Coiled-coil is a common and important protein-protein interaction motif, that consists of two or more α-helices that wrap around each other with a superhelical twist. The coiled-coil of REPS2 might be involved in binding to RALBP1, since RALBP1 binds to the C-terminal side of REPS2 (Ikeda et al., 1998) (see also below).

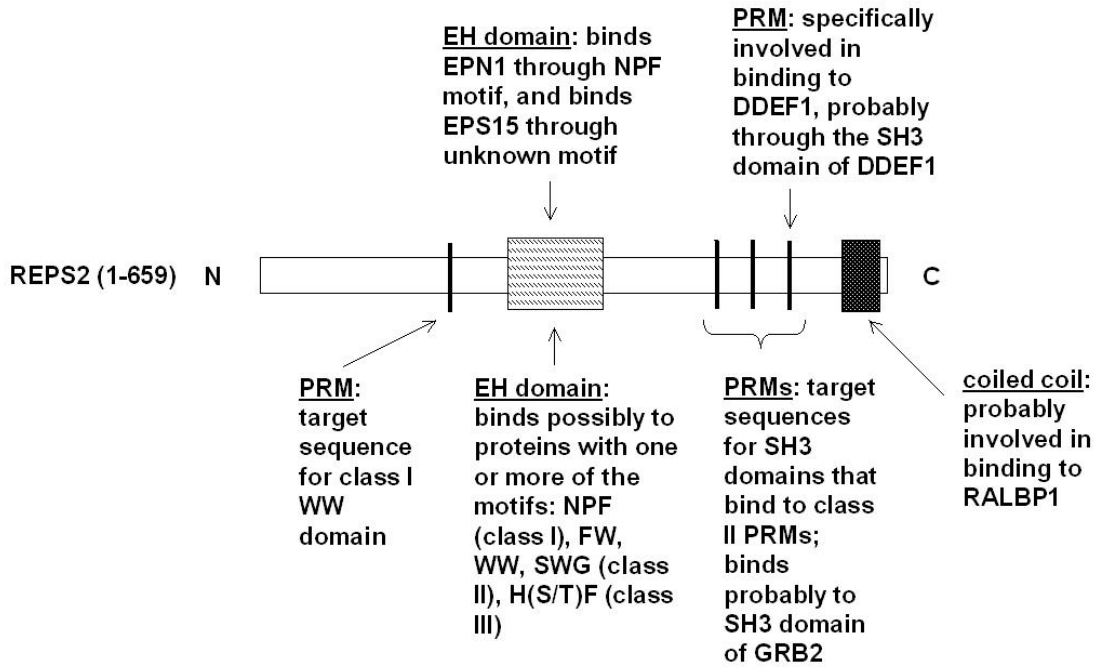

Figure 1 Schematic representation of the REPS2 protein
Regions that are (possibly) involved in protein-protein interaction are indicated. The protein contains: PRM1 (proline-rich motif 1) domain PPSSPPHYQRVP (195-206), EH domain (265-366), PRM2 domain PPTPPPRP (476-483), PRM3 domain PPPPALPPRP (512-521), PRM4 domain PPSKPIR (560-566), coiled-coil domain (607-654). See Paragraph 1.4 for explanation of the protein-protein and domain-motif interactions.

What are the functions for REPS2?

The REPS2 sequence is identical to POB1 (Partner of RALBP1), except that POB1 has an additional glutamine residue inserted between amino acids 181 and 182. The remainder of this paragraph gives a brief overview of the roles that have been described in literature for POB1. Note: POB1 has also been renamed to REPS2, and therefore POB1 is called REPS2.

A role for REPS2 downstream of RAL

RAL (v-ral simian leukemia viral oncogene homolog) proteins are small G proteins, which alter, upon stimulation, from an inactive GDP-bound form to an active GTP-bound form. The RAL guanine nucleotide dissociation stimulator (RALGDS) stimulates this GDP-GTP exchange. RAL-GTP can be reverted to RAL-GDP by RALGAPs (RAL GTPase activating proteins) (Bos, 1998; Feig et al., 1996). RALGAPs accelerate the very low intrinsic GTPase activity of RAL proteins, what leading to hydrolysis of bound GTP and to regeneration of RAL-GDP. RAL proteins are located downstream of the well-known RAS (rat sarcoma viral oncogene homolog) proteins, which are also small G proteins. Besides the RAL pathway, two other pathways have been identified downstream of RAS, namely the mitogen activated protein (MAP) kinase pathway, starting with the MAP kinase kinase kinase RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1), and the PI3K (phosphoinositide-3-kinase) pathway (Shields et al., 2000). In three separate studies to elucidate functions for RAL, RALBP1 (RALA binding protein 1; RALA is a member of the RAL family) was identified as a protein that binds specifically to the active GTP-bound form of RALA (Cantor et al., 1995; Jullien-Flores et al., 1995; Park & Weinberg, 1995). In addition to the RAL-binding domain, RALBP1 also contains a RHO-GTPase-activating protein (RHO-GAP) domain. With this RHO-GAP domain, RALBP1 could activate the intrinsic GTPase activity of the RHO (ras homolog gene family) family members CDC42 (cell division cycle 42) and RAC1 (ras-related C3 botulinum toxin substrate 1), but not RHOA (ras homolog gene family, member A) (Cantor et al., 1995; Jullien-Flores et al., 1995; Park & Weinberg, 1995). The RALBP1-activated intrinsic GTPase activity in CDC42 and RAC1 results in hydrolysis of GTP to GDP in CDC42 and RAC1, and this turns active forms of CDC42 and RAC1 into inactive forms. Therefore RALBP1 action can inhibit the activity of CDC42 and RAC1.
To gain more insight into RALBP1, the protein has been used as bait in a yeast two-hybrid screen. This screen resulted in the isolation of REPS2 as a protein that interacts to RALBP1 (Ikeda et al., 1998). REPS2 bound to the C-terminal region of RALBP1, which is another region to where RALA binds. In COS cells, a ternary complex of RALA, REPS2 and RALBP1 could be demonstrated (Ikeda et al., 1998). Furthermore it was demonstrated that binding of REPS2 to RALBP1 did not affect the RHO-GAP activity of RALBP1 (Ikeda et al., 1998). The pathway is shown schematically in Figure 2.

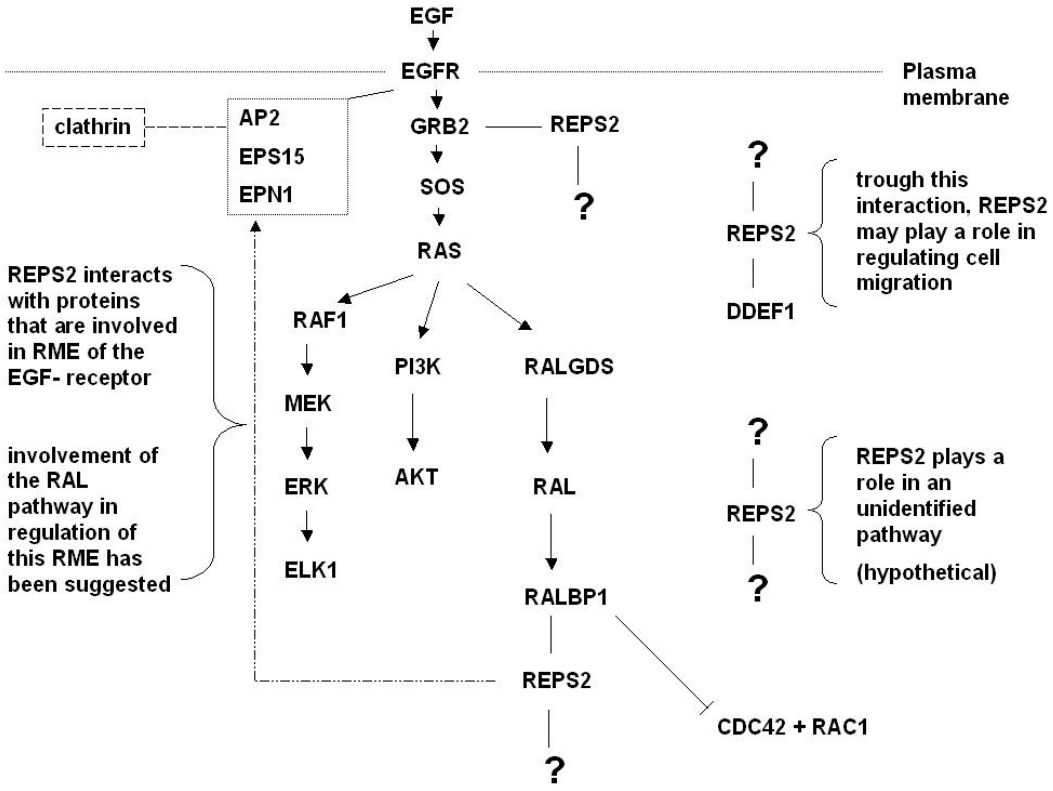

Figure 2 Scheme indicating cellular roles for REPS2
For explanation of abbreviations and interactions, see Paragraph 1.4 (and List of Abbreviations). The question marks represent unidentified REPS2 protein partners.

A role for REPS2 directly downstream of the EGF receptor

The epidermal growth factor (EGF) receptor (EGFR) is a cell surface receptor of the receptor tyrosine kinase (RTK) family. Like all RTKs, the EGF receptor comprises an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane α helix, and a cytosolic domain that includes a region with protein tyrosine kinase activity. The EGF receptor can transmit signals from outside the cell into the cell. One of the targets for the EGF receptor inside the cells is the small GTPase RAS. Binding of EGF to the ligand-binding site induces a conformational change in the receptor that promotes dimerization of the receptor. After dimerization the kinase activity of each subunit of the dimeric receptor initially phosphorylates tyrosine residues near the catalytic site in the partner subunit. Subsequently tyrosine residues in other parts of the cytosolic domain are autophosphorylated (Panayotou & Waterfield, 1993). Growth factor receptor-bound protein 2 (GRB2), which is an adapter protein, binds to a specific phosphotyrosine on the activated EGF receptor. GRB2 binds also to a protein named SOS (Son of Sevenless), and SOS binds to RAS-GDP, the inactive form of RAS. The guanine nucleotide-exchange factor (GEF) activity of SOS then promotes formation of active RAS-GTP (Buday & Downward, 1993; Egan et al., 1993; Lowenstein et al., 1992; Olivier et al., 1993; Simon et al., 1993). The pathway is shown schematically in Figure 2.

REPS2 is able to bind GRB2 (Ikeda et al., 1998), and it is presumably the SH3 (sarc homology 3) domain of GRB2 that binds to proline rich motifs of REPS2 (see also Figure 1). REPS2 may form a complex with the EGF receptor through GRB2, since the EGF receptor is co-immunoprecipitated with REPS2, in extracts from EGF stimulated cells and not from unstimulated cells (Ikeda et al., 1998). Furthermore, tyrosine phosphorylation of REPS2 in response to EGF has been demonstrated in COS cells (Ikeda et al., 1998). Taken together, these data suggest that the REPS2 protein may play a role downstream of the EGF receptor (Figure 2).

A role for REPS2 in ligand-dependent receptor mediated endocytosis

Receptor-mediated endocytosis (RME) is defined as the movement of specific proteins, which cannot pass across the plasma membrane into a cell, by the inward budding of membrane vesicles containing proteins with binding sites for the endocytosed proteins. RME enables cells to decrease the number of growth factor (GF) receptors, e.g. the EGF receptor, on the cell surface. This phenomenon is termed receptor down-regulation (Sorkin & Waters, 1993).
As mentioned before, binding of EGF to the ligand-binding site of the EGFR induces a conformational change in the receptor that promotes dimerization of the receptor and results in an active EGF receptor complex, and this leads ultimately to activation of several signal transduction cascades. In addition, activation of the EGFR by EGF leads to rapid internalization of the receptor. EGF-dependent internalization requires tyrosine phosphorylation of a specific region of the receptor. Clathrin-associated adapter-related protein complex (AP2), which is recruited from the cytosolic pool to the plasma membrane, interacts directly with this tyrosine-based recognition sequence in the cytosolic tail of EGF receptor (EGFR) (Ohno et al. 1995; Sorkin 1996). AP2 is a heterotetrameric protein complex that mediates aggregation of EGF receptors in clathrin-coated pits, through its ability to bind both to the tyrosine-based recognition sequence of the receptor and to clathrin. Subsequently a clathrin-coated vesicle buds off from the plasma membrane. After internalization into the endosomal compartment, a significant pool of EGF and EGFR escape recycling to the cell surface, and are sorted to a degradation pathway (Sorkin & Waters, 1993).
Proteins that contain an EPS15 homology (EH) domain, and proteins that bind to these proteins, are considered to be involved in regulating various steps of endocytosis (Santolini et al., 1999). EGFR pathway substrate 15 (EPS15) (Fazioli et al., 1993) itself has three EH domains. EPS15 plays an important role in receptor-mediated endocytosis (Salcini et al., 1999). For example, clathrin-coated pit assembly was found to be inhibited by an EPS15 mutant (Benmerah et al., 1999). REPS2 also contains an EH domain (see also Figure 1), and therefore the REPS2 protein might play a role in RME. Indeed, literature data suggest that REPS2 plays a role in RME. For example, deletion mutants of REPS2 inhibited internalization of EGF and insulin (Nakashima et al., 1999). Furthermore it has been demonstrated that the EH domain of REPS2 binds directly to EPS15 and EPN1 (Epsin 1) (Morinaka et al., 1999; Nakashima et al., 1999). EPN1 is another protein that is implicated in clathrin-mediated endocytosis (Chen et al., 1998b; Ford et al., 2002). EPN1 has three copies of the asparagine-proline-phenylalanine (NPF) motif in its C-terminal region, which is known to be the major EH domain-binding motif (de Beer et al., 2000; Paoluzi et al., 1998; Salcini et al., 1997) (see also Figure 1). The EH domain of REPS2 has been found to interact directly with the region containing the NPF motifs of EPN1 (Morinaka et al., 1999). Additionally, in another study it was demonstrated that

RALBP1, REPS2, EPN1, and EPS15 form a complex with AP2A (adaptor-related protein complex 2, alpha subunit; formerly known as α-adaptin)(Kariya et al., 2000). Phosphorylation of EPN1 inhibited the binding of EPN1 to the EH domain of REPS2 (Kariya et al., 2000). It has been suggested that phosphorylation of EPN1 in M (mitotic) phase inhibits RME by disassembly of its complex with REPS2 and AP2A (Kariya et al., 2000). Interestingly, in a recent study it was demonstrated that mitotic phosphorylation of EPN1 is dependent on RAL signalling (Rosse et al., 2003).
RME can occur in a ligand-dependent and in a ligand-independent way. Endocytosis of the EGFR occurs in both ways, whereas RME of the transferrin receptor occurs in a ligand-independent way only. The RAL/RALBP1/REPS2 pathway might be involved in ligand-dependent RME, since mutated forms of RAL, RALBP1, and REPS2 can block internalisation of the EGFR but not internalisation of the transferrin receptor (Nakashima et al., 1999). This observation, together with the identified REPS2-EPN1 and REPS2-EPS15 interactions, and the fact that EGF is able to activate RAL, through RAS (Wolthuis et al., 1998), has led to the suggestion that RAL, RALBP1, and REPS2 transmit the (EGF) signal from the receptor to EPN1 and EPS15, and thereby regulate ligand-dependent receptor mediated endocytosis (Nakashima et al., 1999) (Figure 2).

A role for REPS2 in cell migration

In a recent paper, DDEF1 (development and differentiation enhancing factor 1; formerly known as PAG2) was described as a novel binding partner for REPS2 (Oshiro et al., 2002). DDEF1 is a paxillin-associated protein with ADP-ribosylation factor GTPase-activating protein activity. The DDEF1 protein has similarity with proteins from the ARFGAP (ADP-ribosylation factor GTPase activating protein) family (Randazzo & Hirsch, 2004; Turner et al., 2001). These proteins can negatively regulate the activity of ARF (ADP-ribosylation factor) proteins, which are small G proteins that are implicated in membrane trafficking and actin remodeling (Donaldson & Jackson, 2000; Randazzo et al., 2000). All ARFGAP proteins have a conserved ARFGAP domain, which contains a zinc finger motif (Cukierman et al., 1995). Besides the ARFGAP domain, the DDEF1 protein has several well-known domains, and some of theses domains might play a role in protein-protein interaction. Ordered from the N-terminus to the C-terminus, the protein contains a pleckstrin homology (PH) domain, the ARFGAP domain with the zinc finger motif, an ankyrin (ANK) repeat, three proline-rich motifs (PRMs) and a sarc homology 3 (SH3) domain. It was demonstrated that the SH3 domain of DDEF1 binds the most C-terminal proline-rich motif in REPS2 (Oshiro et al., 2002) (see also Figure 1). Mutations in this proline-rich motif of REPS2 impaired the binding between REPS2 and DDEF1. Experiments have been described that show functional interaction of REPS2 with DDEF1. It was demonstrated that expression of DDEF1 inhibits fibronectin-dependent migration and also recruitment of paxillin to focal contacts of CHO-IR cells (Oshiro et al., 2002). Co-expression with REPS2 suppressed this inhibitory action of DDEF1 on cell migration and paxillin localization. However, REPS2 mutated in the proline-rich motif, was unable to suppress this inhibitory action of DDEF1 (Oshiro et al., 2002). Taken together, REPS2 is able to bind DDEF1 through its most C-terminal located proline-rich motif, and this interaction may regulate cell migration (Figure 2).

1.5 Aim and outline of this thesis

The observed decrease of the REPS2 mRNA level in AIPC (Paragraph 1.4) probably leads to a decrease of REPS2 protein level in AIPC. As a consequence, this may result in loss of different protein-protein interactions which require REPS2. Some of these lost protein-protein interactions might be important for the transition from androgen-dependent to androgen-independent prostate cancer.

The possible role of REPS2 downstream of the EGF receptor (EGFR) (Paragraph 1.4), and the observation that REPS2 has an EH domain (Paragraph 1.4), which suggests that REPS2 plays a role in endocytosis, has led at the beginning of the project to the following working hypothesis:

REPS2 forms a protein bridge between an activated growth factor receptor and an adaptor complex, and parts of the growth factor receptor internalisation and intracellular trafficking machinery. In other words, REPS2 is involved in growth factor receptor down-regulation. When expression of REPS2 is low, as in androgen-independent prostate cancer, an activated growth factor receptor will no longer be downregulated and can maintain its growth stimulatory action.

This might be a mechanism through which prostate cancer cells can escape apoptotic cell death, induced by androgen ablation. The importance of such a mechanism is illustrated by results from Wells et al. (1990) and Masui et al. (1991), who show that noninternalizing epidermal growth factor receptors have high oncogenic potential.

The aim of the work described in this thesis was to identify new cellular binding partners for REPS2. These newly identified binding partners can play a role in RME, which would support the working hypothesis (see above) or may reveal other cellular functions for REPS2. The information obtained may elucidate roles for REPS2, which may explain why loss of REPS2 involved protein-protein interaction leads to additional cell survival in AIPC.

REPS2 mRNA is downregulated in androgen-independent prostate cancer compared to androgen-dependent prostate cancer. This decrease in mRNA level does not necessarily mean that REPS2 protein level is also decreased in AIPC. With specific antibodies against REPS2 it is shown that the REPS2 protein level indeed is in agreement with the REPS2 mRNA level, both in ADPC and AIPC (**Chapter 2**). In this Chapter it is also shown that REPS2 occurs in a shorter and a longer form, and overexpression of both forms induces apoptosis in androgen dependent LNCaP-FGC prostate cancer cells. This induction of apoptosis shows that loss of REPS2 protein may indeed lead to additional cell survival. To elucidate cellular functions of REPS2, proteins were identified that bind REPS2. Therefore REPS2 was used as bait in a yeast two-hybrid screen. In this screen several human protein sequences bound physically to REPS2 in yeast cells (**Appendix**). One prey that bound to the REPS2 bait represents a large fragment of the NF-κB subunit p65 (RELA) (**Chapter 3**). p65 promotes cell survival and possibly plays an important role in prostate cancer progression, because p65 is inactive in ADPC but active in AIPC. The interaction of REPS2 with p65 is studied in more detail (**Chapter 3**). Additional data on the REPS2-p65 interaction, including the effect of REPS2 expression on the NF-κB

pathway is described in **Chapter 4**. This Chapter describes also observed interactions between REPS2 and other proteins, including TRAF4 and STAT6.
The identified REPS2 protein partners (**Appendix**) seem not to play a prominent and direct role in regulating ligand-induced RME of growth factor receptors. Therefore our working hypothesis (see above) is not supported by the present results. However, p65, TRAF4 and STAT6, three of the identified REPS2 protein partners suggest a role for REPS2 in regulating cell survival. In **Chapter 5** it is discussed that such a new role for REPS2 might be implicated in development of androgen-independent prostate cancer, following the androgen-dependent stage.

References

References are listed on pages 88-93.

CHAPTER 2

REPS2/POB1 is downregulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells

REPS2/POB1 is downregulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells

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The nucleotide sequences reported in this paper have been submitted to GenBank under accession number AF511533 (REPS2a) and AF512951 (REPS2b)

Abstract

During progression of prostate cancer, cellular changes occur, leading to a transition from androgen-dependent to androgen-independent growth. One aspect of this transition is a switch from androgens to growth factors, like epidermal growth factor (EGF), as primary regulators of proliferation. We examined the involvement of REPS2/POB1 in this process. REPS2/POB1 is an EH domain-containing protein, reported to be involved in signalling via RalBP1 and to play a role in endocytosis of EGF receptors. Furthermore, the protein is relatively highly expressed in androgen-dependent as compared to androgen-independent human prostate cancer cell lines and xenografts. Next to the known REPS2/POB1 protein, an open reading frame encoding REPS2/POB1, with 139 additional amino-acid residues at the NH_2-terminus, was cloned and found to be expressed in prostate cancer cells. Overexpression, by transient transfection, of both forms of REPS2/POB1 in prostate cancer cell lines, induced apoptosis within 48 h. At shorter time intervals after transfection, signalling towards a TPA response element luciferase reporter was found to be inhibited. From these experiments, it is concluded that REPS2/POB1, through its influence on the Ral signalling pathway, is involved in growth factor signalling. Decreased expression of REPS2/POB1 during progression of prostate cancer may therefore result in loss of control of growth factor signalling and consequently in loss of control of cell proliferation.

Introduction

The transition from androgen-dependent to androgen-independent growth during prostate cancer progression is of great concern, since androgen-independent tumours are incurable. One possible mechanism underlying this transition is a switch from androgens to growth factors as primary regulators of prostate cancer cell proliferation. Fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and epidermal growth factor (EGF) are examples of autocrine factors which stimulate growth of advanced, androgen-independent, prostate cancer (Russell et al., 1998). For EGF, binding of the ligand to the receptor induces cell proliferation by activating well-characterized signal transduction pathways that involve phosphorylation and activation of mitogen-activated protein kinases (MAPKs). The activation of the GTP-ase Ras is one of the initial steps in EGF signalling, and once Ras is activated, it can facilitate activation of the serine/threonine kinase Raf, PI3-kinase and Ral-GEF, a guanine nucleotide exchange factor that activates the small GTP-ase Ral (reviewed by Pruitt & Der, 2001). Activated Ral binds to RalBP1, which exhibits GAP (GTPase-activating protein) activity towards Cdc42 and Rac (Cantor et al., 1995; Jullien-Flores et al., 1995; Park & Weinberg, 1995).
REPS2/POB1 is a protein partner of RalBP1, contains a single EH domain, two SH3 binding sites and several other functional regions (Ikeda et al., 1998). REPS2/POB1 has been implicated in endocytosis and signal transduction (Ikeda et al., 1998; Kariya et al., 2000; Nakashima et al., 1999) and is currently evaluated for its putative role in prostate cancer progression.

Endogenous expression of REPS2/POB1 in prostate cancer cell lines

In a previous study, reduced mRNA expression of REPS2/POB1 was found in the androgen-independent human prostate cancer cell lines PC3, DU145 and LNCaP-LNO, as compared to the expression level in the androgen-dependent LNCaP-FGC cell line. In addition, using a panel of human prostate cancer xenografts, REPS2/POB1 expression was also found to be markedly reduced in androgen-independent prostate cancer xenografts (Chang et al., 1997).
Using a hypothalamus cDNA library, a 1987 bp cDNA clone was obtained containing an open reading frame encoding a protein of 659 amino-acid residues. This protein consists of the published coding region of REPS2/POB1 (accession number AF010233) minus one glutamine at position 43, and 139 additional amino-acid residues at the NH_2-terminal end (Figure 1a). All reported studies so far, however, made use of the shorter 521 amino-acid REPS2/POB1 protein. We examined the possible translation of the extended REPS2/POB1 protein in human cells using a specific REPS2/POB1 antibody and were able to detect 78 and 58 kDa protein bands in lysates from prostate cancer cell lines (Figure 1b). These results indicate that both the 659 amino-acid residue and the 521 amino-acid residue REPS2/POB1 protein are expressed in these cell lines.

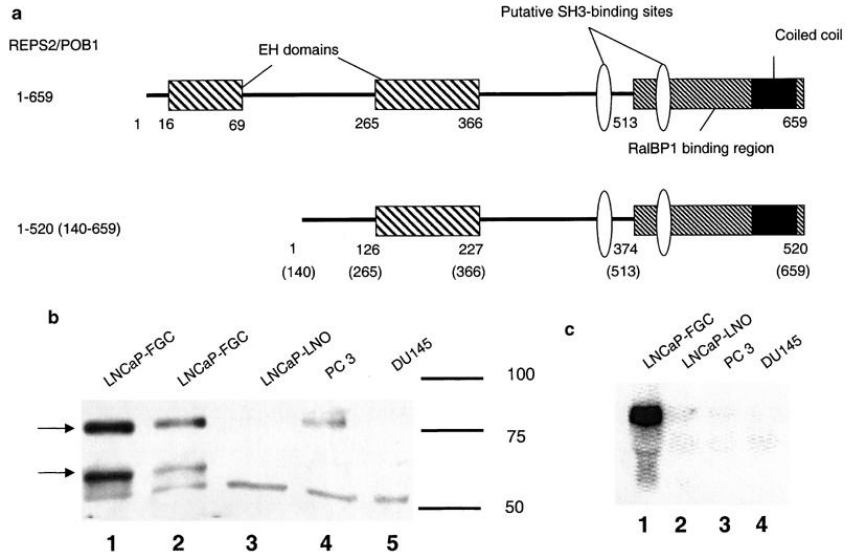

Figure 1 Analysis of endogenous REPS2/POB1 expression in prostate cancer cell lines: The LNCaP-FGC (passage number 22–30) and LNCaP-LNO (passage number 78–100) cell lines were kindly provided to us by Dr JS Horoszewicz (Buffalo, NY, USA). The LNCaP-FGC cell line is identical to the one which can be obtained from the American Type Culture Collection (Rockville, MD, USA). LNCaP-FGC, LNCaP-LNO, PC3 and DU145 cells were maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 plus 5% v/v fetal calf serum (FCS) or serum depleted of steroids by dextran-coated-charcoal (DCC) treatment (van Steenbrugge et al., 1991). The LNCaP-LNO cell line has been derived from an early passage (sixth) of the parental LNCaP-FGC cell line (Horoszewicz et al., 1980). (**a**) Linear map of REPS2/POB1 (1–659) and REPS2/POB1 (140–659), indicating the relative location of EH domains, proline-rich regions, RalBP1 binding region and predicted coiled coil (adapted from Ikeda et al., 1998). (**b**) Western blot analysis of lysates of LNCaP-FGC passage numbers 26 and 28 (lanes 1 and 2), LNCaP-LNO (lane 3), PCS (lane 4) and DU 145 (lane 5) after 7% SDS–PAGE, showed REPS2/POB1 downregulation in androgen-independent prostate cancer cell lines. The antiserum was generated by immunizing rabbits with two REPS2/POB1 peptides; peptide 1: amino-acid residues 446–461 and peptide 2: amino-acid residues 566–580, and then affinity-purified. The arrows point at the two REPS2/POB1 protein forms. (**c**) Northern blot analysis of REPS2/POB1 mRNA expression in prostate cancer cell lines. Total RNA of LNCaP-FGC (lane 1), LNCaP-LNO (lane 2), PC3 (lane 3) and DU 145 (lane 4) was hybridized with a REPS2/POB1 cDNA probe.

Another argument in favour of the coexistence of a longer REPS2/POB1 protein, is the observation that the translation start sequence, proposed by Kozak (Kozak, 1987), is more consistent with the sequence around the more 5' ATG, described herein, than the translation start sequence of REPS2/POB1 described by Ikeda et al. (1998) (Figure 2a). Furthermore, the additional 139 NH_2-terminal amino-acid residues form a putative second EH domain (Figure 2b) and this amino-acid sequence exhibits high homology with the mouse Reps1 and human REPS1 proteins (72% identity). Whether both REPS2/POB1 products are formed by alternative first ATG usage or alternative pre-mRNA splicing is currently unknown.

a

```
Kozak sequence                    CCGCCG/ACCATGG
                                  | | |   ||||||   Identity = 75%
First ATG of REPS2/POB1(1-659)    CTGGC C CCATGG

Kozak sequence                    CCGCCG/ACCATGG
                                  |   |     |||    Identity = 42%
First ATG of REPS2/POB1 (140-659) CTCGC T TTATGA
```

b

```
REPS2_EH1 ............................... 0
REPS2_EH2 ...WRITEEQREYYVNQFRSL.QPDPSSFI 26
Eps15_EH3 ...WVVSPAEKAKYDEIFLKTDKDMDGF.V 26
Eps15R_EH ....PTGNSLYESYYKQVDPA.Y..TGR.V 22
Inters_EH1..MWAITSEERTKHDRQFDNL.KPSGGY.I 26
Eps15_EH1 LTQLSSGNPVYEKYYRQVDTG.N..TGR.V 26
Consensus    W       EKY  QFD       G  V

REPS2_EH1 AAGPVADLFRASQLPAETLHQITELCGAKR 30
REPS2_EH2 SGSVAKNFFTKSKLSIPELSYIWELSDADC 56
Eps15_EH3 SGLEVREIFLKTGLPSTLLAHIWSLCDTKD 56
Eps15R_EH GASEAALFLKKSGLSDIILGKIWDLADPEG 52
Inters_EH1TGDQARNFFLQSGLPAPVLAEIWALSDLNK 56
Eps15_EH1 LASDAAAFLKKSGLPDLILGKIWDLADTDG 56
Consensus  AS AA FF KSGLP   L  IW L D

REPS2_EH1 VGYFGPTQFYIALKLIAAAQS.GLPVRIES 59
REPS2_EH2 DGALTLPEFCAAFHLIVARKN.GYPLPEGL 85
Eps15_EH3 CGKLSKDQFALAFHLISQKLIKGIDPPHVL 86
Eps15R_EH KGFLDKQGFYVALRLVACAQS.GHEVTLSN 81
Inters_EH1DGKMDQQEFSIAMKLIKLKLQ.GQQLPVVL 85
Eps15_EH1 KGILNKQEFFVALRLVACAQN.GLEVSLSS 85
Consensus  GL  KGEF  AL LIA AQ  G  VP  L

REPS2_EH1  IKCELPLPRF                    69
REPS2_EH2  PPTLQ                         90
Eps15_EH3  TPEMI                         91
Eps15R_EH  LNLSMPPPKF                    91
Inters_EH1 PPIMKQPP                      93
Eps15_EH1  LNLAVPPPRF                    95
Consensus   P   PPP F
```

Figure 2 Sequence properties of REPS2/POB1 cDNA: (**a**) Comparison of translation start site of REPS2/POB1 and REPS2/POB1 (140–659) with the start site proposed by Kozak (1987). (**b**) Alignment of several EH domains. REPS2_EH1 is the EH domain found in the first 139 amino-acid residues of REPS2/POB1. Also presented are: REPS2_EH2: REPS2/POB1 (275–364); Eps15_EH3: Eps15 (218–313); Eps15R_EH: Eps15R (13–103); lnters_EH1: Intersectin2 (28–118) and Eps15_EH1: Eps15 (16–103). Amino acids shared between more than 50% of the EH domains are in italics, between more than 75% are underlined and 100% alignment is shown in bold. All sequences are from human. Sequences were aligned using the DNAMAN program.

We observed a good correlation between mRNA and protein expression of REPS2/POB1 in DU145, PC3, LNCaP-FGC and LNCaP-LNO cells. The LNCaP-FGC cells were found to express REPS2/POB1 mRNA and protein at relatively high levels, whereas LNCaP-LNO and DU145 cells do not express detectable levels of REPS2/POB1 mRNA and protein. The PC3 cells show low expression of REPS2/POB1 protein and no detectable expression of REPS2/POB1 mRNA (Figure 1b,c).

Role of REPS2/POB1 in signal transduction: apoptosis

To determine the cellular localization of REPS2/POB1 in prostate cancer cells, LNCaP-FGC and LNCaP-LNO cells were transiently transfected with cDNA encoding REPS2/POB1 or REPS2/POB1 (140–659) fused to GFP. Cells were analysed with fluorescence microscopy at different time points after transfection. It was observed that both variants of REPS2/POB1 mainly reside in the cytosol and not in the nucleus (Figure 3a). Subsequently, the cells were subjected to prolonged expression of GFP-tagged REPS2/POB1, REPS2/POB1 (140–659) or part of the androgen receptor (AR), to examine the effect of REPS2/POB1 and REPS2/POB1 (140–659) on prostate cancer cell growth. The tagged AR was used to evaluate nonspecific effects of the transfection procedure. REPS2/POB1 was found to induce programmed cell death. Transfected cells that showed DNA fragmentation and cell membrane damage, as observed using Hoechst and propidium iodide staining, were considered to be apoptotic (Figure 3b-d). REPS2/POB1 (140–659) or REPS2/POB1-induced apoptosis in 45% of transfected cells already within 48 h and up to 60% after 96 h (Figure 3e). Based on these observations, a regulating role for REPS2/POB1 in a signalling pathway that controls apoptosis was hypothesized.

We investigated whether the role which REPS2/POB1 seems to play in induction of apoptosis can be explained when the function of one of its binding partners, RalBP1, is implicated. When we transfected LNCaP cells with a deletion mutant of REPS2/POB1 lacking the RalBP1 binding domain (1–373), only 30–40% of the transfected cells became apoptotic (72–96 h), while a mutant containing only the RalBP1 binding domain of REPS2/POB1 (451–659) induced apoptosis in 40–75% of the transfected cells (Figure 3f). Since the expression levels of the two proteins were similar, these observations suggest that the RalBP1 binding properties of REPS2/POB1 are at least partly responsible for apoptosis induction in these cells.

RalBP1 is a protein with GTPase activating protein (GAP) activity towards Rac1 and Cdc42 (Cantor et al., 1995; Jullien-Flores et al., 1995; Park & Weinberg, 1995). Activated Rac1 and Cdc42 have been implicated in signalling towards cell survival (Joneson & Bar-Sagi, 1999; Osada et al., 1999) and, because of its GAP activity, RalBP1 is capable of inhibiting these functions. The GAP activity of RalBP1, in turn, can be inhibited by activated Ral, which translocates RalPB1 to the cell membrane and physically away from Rac1 and Cdc42 (Matsubara et al., 1997). The presence of REPS2/POB1, as a cytosolic protein, may in this respect function to counteract activated Ral, maintaining a number of RalBP1 molecules in the cytosol and thus available to inactivate Rac1 and Cdc42. Overexpression of REPS2/POB1 may result in a strong inhibition of Rac1 and Cdc42 signalling, which may consequently result in the observed induction of apoptosis.

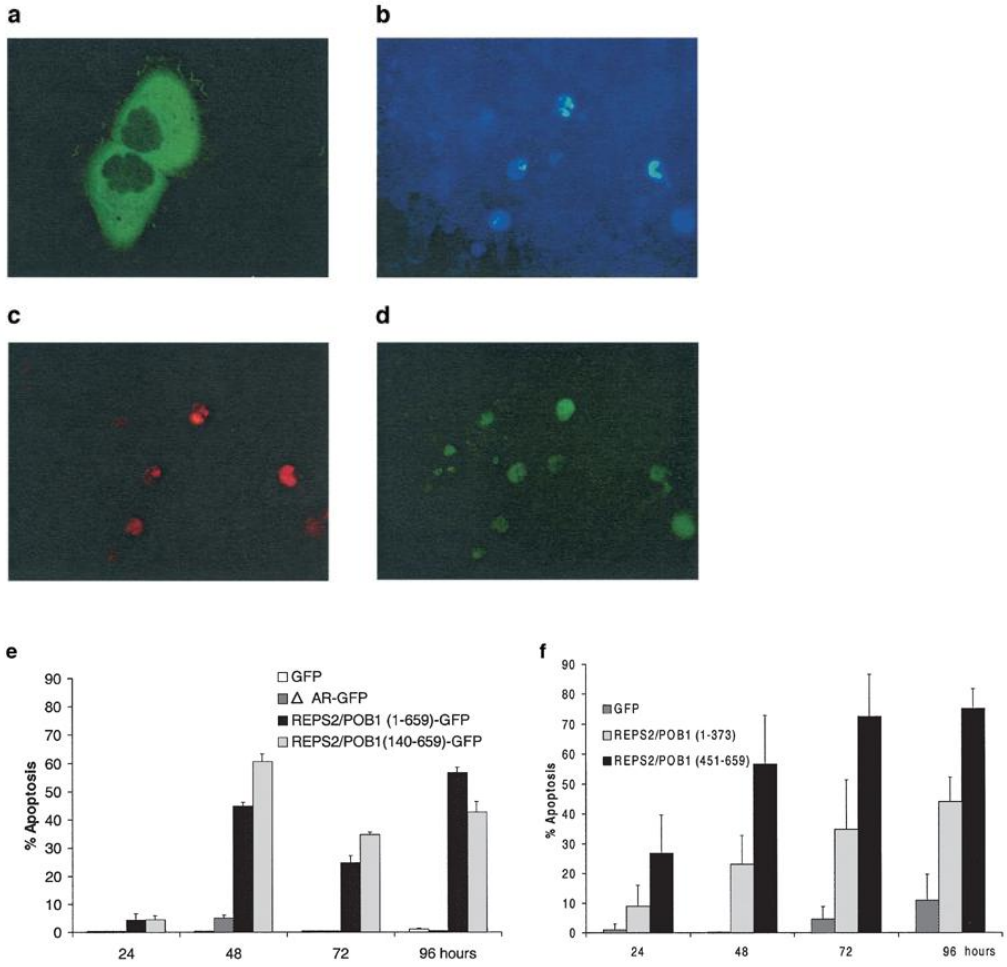

Figure 3 Induction of apoptosis after transfection with REPS2/POB1-GFP
REPS2/POB1 and REPS2/POB1 (140-659) cDNA was obtained from a human brain, thalamus 5'-stretch plus cDNA library (Clontech) and cloned into the pEGFP-N_2 vector (Clontech) between the Xho1 and Kpn1 sites. Deletion mutant REPS2/POB1 (1-373) and REPS2/POB1 (451-659) were synthesized by PCR and ligated in pEGFP-N_2 pEGFP-ΔAR consists of a *Hind*III fragment of the cDNA encoding the NH_2-terminal part of the AR, cloned into pEGFP-N_2. LNCaP-FGC cells transfected with cDNA encoding REPS2/POB1-EGFP, ΔAR-EGFP or EGFP alone (1 μg/ml medium) were analysed by fluorescence microscopy. (a) For localization of the REPS2/POB1-EGFP product, LNCaP cells transfected with pEGFP-RESP2/POB1 were studied using confocal laser scanning microscopy. LNCaP-FGC cells transfected with REPS2/POB1-EGFP show cytoplasmic localization of REPS2/POB1-EGFP (magnification: ´ 100). Analysis after 72 h of transfection with REPS2/POB1-EGFP with fluorescence microscopy. (b) Hoechst 33342 staining, (c) propidium iodide staining, (d) REPS2/POB1-EGFP expression (magnification: ´ 40). (e) Cells with positive Hoechst and propidium iodide fluorescence were quantified as a percentage of EGFP-positive cells at different time points after transfection. (f) Apoptosis induction by deletion mutant constructs of REPS2/POB1 fused to GFP quantified as a percentage of EGFP-positive cells, at different time points after transfection. The results shown are the mean ± s.d. of three independent experiments; each experiment included two independent culture plates.

The deletion mutant that lacks the RalBP1 binding domain also induces apoptosis, but less efficient, suggesting that the two EH domains of REPS2/POB1 may also play a role. The C-terminal EH domain of REPS2/POB1 binds to Eps15 and Epsin and is involved in internalization of growth factor receptors (Nakashima et al., 1999). Disruption of endocytosis might lead to improper signal transduction and consequently to cell death. For example, it has been reported that inhibition of endocytosis of EGF receptors or insulin receptors by a dominant interfering mutant of dynamin results in inhibition of the activation of the protein kinases ERK1 and ERK2, emphasizing the importance of signalling during and after endocytosis (Ceresa et al., 1998; Vieira et al., 1996).

Role of REPS2/POB1 in signal transduction: inhibition of signalling

It has been described that Rac1 and Cdc42 are involved in activation of Jun (Coso et al., 1995) and heterodimers of Jun and Fos activate, among others, genes controlled by promoter sequences containing a TPA response element (TRE) (Angel et al., 1987). A phorbolester responsive reporter system (5 × TRE luciferase reporter) was used to investigate the effect of transient REPS2/POB1 expression in the human prostate cancer cell line PC3. The TRE reporter can be activated by several growth factors (including EGF), serum and synthetic compounds like phorbol esters. Since PC3 cells are not very sensitive to EGF (Janssen et al., 1995), 15% v/v fetal calf serum was used to activate transcription of the luciferase reporter gene. It was observed that luciferase transcription was increased approximately eight- to 12-fold over control expression by addition of serum. Furthermore, short-term transfection (24 h) with increasing amounts of REPS2/POB1 cDNA/well inhibited serum-induced activation of the TRE reporter. Using 10, 30 and 100 ng REPS2/POB1 cDNA/well, a 46, 80 and 90% reduction in luciferase activity was observed, respectively, compared to mock-transfected cells (Figure 4). This reduction in luciferase activity was not a result of REPS2/POB1-induced apoptosis and a decreasing cell number, because apoptosis is not observed at 24 h after transfection. Furthermore, in a parallel transfection assay, where a RSV-luciferase reporter was used, no significant inhibition of luciferase activity was observed with increasing amounts of REPS2/POB1 cDNA. These results indicate that overexpression of REPS2/POB1 exerts a marked inhibitory effect on serum-induced signalling to a TRE reporter.
In advanced prostate cancer cells, the expression status of REPS2/POB1 does not represent overexpression, but rather loss of expression. Consequently, the inhibiting activity of RalBP1 on Rac1 and Cdc42 may be compromised, resulting in loss of control of cell survival and consequently mitogenic signalling.

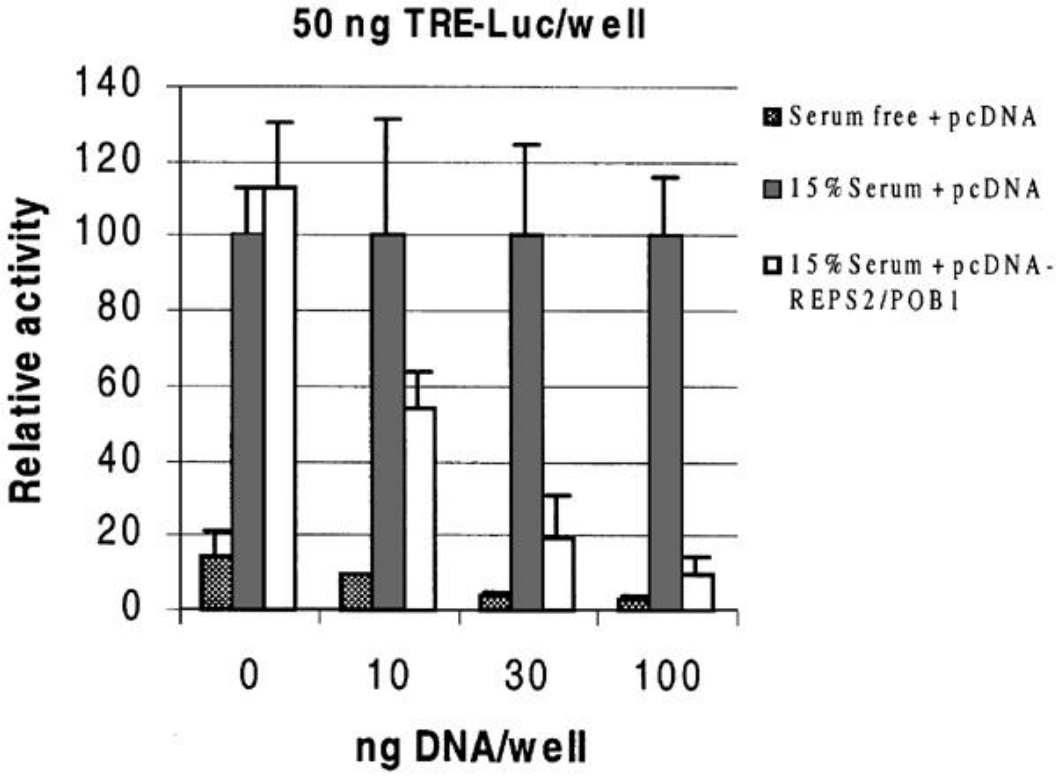

Figure 4 Effect of REPS2/POB1 on TRE-Luc activation in PC3 cells: pcDNA3.1-REPS2/POB1 was constructed from pEGFP-RESP2/POB1. REPS2/POB1 cDNA was cloned into pcDNA3.1 (Invitrogen) between the *Nhe*1 and *Kpn*1 sites. 5 × TRE-TATA-Luc was constructed from 5 × TRE-TATA-CAT6 (a gift from Dr A Cato, Karlsruhe, Germany). PC3 cells were cotransfected with 50 ng TRE-Luc plasmid DNA, in combination with 0, 10, 30 or 100 ng of psDNA3.1-REPS2/POB1 or the empty pcDNA3.1 vector. Stimulation with 15% FCS was started immediately after the start of transfection, and luciferase activity was determined at 24 h with the Topcount NXT^{TM} microplate luminescence counter (Packard Bioscience BV; Meriden, CT, USA). The total amount of DNA in each transfection was normalized using pTZ19U plasmid (US biochemical). The results are expressed relative to the level of luciferase activity in cells cotransfected with the empty vector and the TRE-Luc vector after stimulation with serum. The mean±s.d. of an experiment performed in triplicate is shown. Similar results were observed in two independent experiments.

It is concluded that loss of REPS2/POB1 expression, which occurs during progression of prostate cancer, results in dysregulation of growth factor signalling. This can be the result of loss of the interaction of REPS2/POB1 with RalBP1 or the result of loss of function of REPS2/POB1 during growth factor receptor internalization. Furthermore, it is also possible that both mechanisms simultaneously play a role in REPS2/POB1-induced dysregulated growth factor signalling. Since RalBP1, like REPS2/POB1, has also been reported to be involved in endocytosis (Jullien-Flores et al., 2000), effects of REPS2/POB1 on endocytosis, via binding to RalBP1, and then on signal transduction, cannot be excluded. Furthermore, it has been described that dysregulated endocytosis can lead to mitogenic signalling, and that mutation or abnormal

expression of endocytic proteins is implicated in tumour development (Di Fiore & Gill, 1999; Floyd & De Camilli, 1998). Further research utilizing stable inducible cell lines expressing REPS2/POB1 at a more physiological level, will be used to unravel the precise role of REPS2/POB1 in signalling and endocytosis.
Taken all data together, there is evidence to suggest that loss of REPS2/POB1 expression, during progression of prostate cancer, results in loss of control of cell growth signalling. This can be a direct effect through RalBP1 or an indirect effect via a function in growth factor internalization. Further investigations of the role of REPS2/POB1 in control of prostate cancer progression are important to improve our understanding of androgen-independent tumour growth.

References

References are listed on pages 88-93.

CHAPTER 3

Identification of REPS2 as a putative modulator of NF-kappaB activity in prostate cancer cells

Identification of REPS2 as a putative modulator of NF-κB activity in prostate cancer cells

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Abstract

The protein REPS2 is implicated in growth factor receptor-mediated endocytosis and signalling, and its expression is downregulated in androgen-independent prostate cancer cells. Herein, the NF-κB subunit p65 is identified as a human REPS2 protein partner, interacting with the EH domain of REPS2. Using crystal structure data from literature and experimental data from yeast and mammalian two-hybrid analysis, the results indicate that the NPF-motif in p65 acts as binding site for the EH domain in REPS2. However, in cultured prostate cancer cells, the REPS2-p65 interaction is triggered upon stimulation with phorbol ester (PMA). This indicates that PMA-sensitive signalling pathways can affect the interaction between REPS2 and p65. During prostate cancer progression from androgen-dependent to androgen-independent growth, downregulation of REPS2 is accompanied by upregulation of NF-κB activity. This might involve loss of REPS2-p65 interaction, which would lead to increased NF-κB activity. Androgen-deprivation causes apoptosis of prostate cancer cells, and activated NF-κB is a known inhibitor of apoptosis. Hence, decreased expression of REPS2 might be a key factor, causing prostate cancer cells to become resistant to induction of apoptosis by androgen deprivation.

Introduction

Growth of advanced prostate cancer initially is controlled by androgen ablation therapy. Reduction of the circulating androgen level to almost zero induces apoptosis of hormone-dependent normal prostate cells, and also induces apoptosis of prostate cancer cells. However, remaining prostate cancer cells progress from androgen-dependent towards androgen-independent growth, within a few years, and this means transition to uncontrollable cancer. To study molecular and cellular mechanisms involved in this transition, we have conducted a differential display PCR between androgen-dependent and androgen-independent prostate cancer cells (Chang et al., 1997). Clone JC19 was isolated as a transcript expressed at a five-fold higher level in an androgen-dependent prostate cancer cell line (LNCaP-FGC), as compared to three androgen-independent prostate cancer cell lines (LNCaP-LNO, PC3 and DU145).

Furthermore, using a panel of androgen-dependent and androgen-independent human prostate cancer xenografts, a similar change in JC19 expression was observed (Chang et al., 1997). Clone JC19 was identified to encode REPS2 (RALBP1-associated Eps domain containing protein 2).
Although REPS2 is differentially expressed between androgen-dependent and androgen-independent prostate cancer cell lines, it is not androgen regulated (Chang et al., 1997). Furthermore, the REPS2 gene is located on the human X chromosome at Xp22, and transcribes into a single mRNA of approximately 7000 base pairs. This mRNA contains a maximum open reading frame (ORF), which encodes a protein of 659 amino acids, but on Western blot two REPS2 proteins were observed (Oosterhoff et al., 2003). The 78 kDa protein, REPS2a, is encoded by the maximal ORF that is present in the REPS2 mRNA. The other protein, REPS2b, is encoded by the same ORF but is shorter, with a molecular mass of 58 kDa and consisting of amino acid residues 140-659, because the second N-terminal methionine is used as a translation start (Oosterhoff et al., 2003). REPS2 was initially named POB1 (Partner Of RalBP1) (Ikeda et al., 1998), and identified as a 521-amino-acid protein that binds to RalBP1 (Rac/CDC42 GTPase-activating protein). The shorter form of REPS2 (REPS2b) is identical to the POB1 protein, except that POB1 has an additional glutamine residue inserted between amino acids 181 and 182.
REPS2 contains at least three different regions potentially involved in protein-protein interactions. First, at the C-terminus there is a coiled-coil protein-protein interaction domain, which is part of a larger region (amino acids 513-659) that is involved in RalBP1 binding (Ikeda et al., 1998). Second, there are two proline-rich motifs, PPTPPPRP (amino acids 476-483) and PPPPALPPRP (amino acids 512-521), which are putative binding sites for proteins containing an Src homology 3 (SH3) domain. The protein Grb2, containing an SH3 domain, has been reported to bind to REPS2 (Ikeda et al., 1998). Third, REPS2 contains one imperfect Eps15 homology (EH) domain (amino acids 16-69) and one consensus EH domain (amino acids 265-366). EH domain proteins are thought to play a role in receptor-mediated endocytosis (Santolini et al., 1999), and for REPS2 it has been demonstrated that the consensus EH domain binds Epsin and Eps15, two proteins that are implicated in endocytosis (Nakashima et al., 1999). In addition, it was shown that deletion mutants of REPS2 can inhibit internalization of EGF and insulin receptors (Nakashima et al., 1999). Based on these observations, REPS2 is thought to be involved in growth factor receptor signalling and internalization. Recently, it was shown that overexpression of REPS2 in prostate cancer cells leads to induction of apoptosis (Oosterhoff et al., 2003), which may point to an additional role for REPS2 in cellular mechanisms other than receptor internalization.
To study molecular and cellular aspects of the possible role of REPS2 in the transition of prostate cancer cells from androgen-dependent to androgen-independent growth in more detail, experiments were performed to identify novel protein partners of REPS2. Using a human prostate cDNA expression library, and yeast and mammalian two-hybrid strategies, REPS2 was found to interact with the NF-κB subunit RelA/p65.

Results

P65 and Clone4 were identified as binding partners of REPS2

To identify proteins that interact with the REPS2 protein, a human prostate cDNA expression library was screened with REPS2a (1-659) as a bait (Figure 1). Herein, we report on two proteins that showed binding to REPS2. One of the prey proteins that bound the REPS2 bait appeared to be a large fragment of the NF-κB subunit RelA/p65 protein (74-551). Since the relevant functional domains of p65 are located within this prey protein, the current clone was used as the basis for our further investigations. The second prey represented a large fragment of a hypothetical protein (GenBank accession number AF448860) and was named Clone4. In the present study, REPS2-Clone4 interactions serve as a control for the REPS2-p65 interaction.

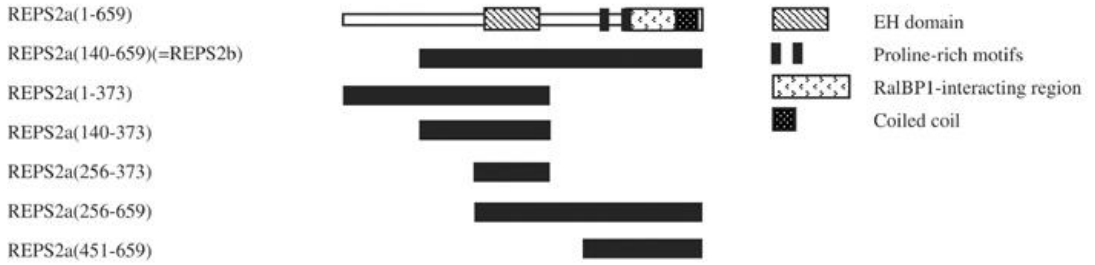

Figure 1 Schematic representation of seven REPS2 variants used in the present study

The REPS2 EH domain binds to p65

To determine which part of REPS2 binds to p65 and Clone4, different REPS2 variants (Figure 1) were used in yeast two-hybrid analysis. Yeast strain AH109 was cotransformed with the REPS2-bait and p65- or Clone4-prey constructs as indicated in Table 1. Equal amounts of the cotransformation mix were plated out on medium that lacked tryptophan and leucine (only transformed cells survive), and on medium that lacked tryptophan, leucine, histidine and adenine (only transformants with interaction between bait and prey survive). When the REPS2 variants were cotransformed with empty prey vector, none of the cotransformants were able to survive the histidine and adenine selection (Table 1a; columns -T-L-H-A). This means that the REPS2 baits alone do not activate the histidine and the adenine marker in the yeast genome. However, when the REPS2 variants were cotransformed with the p65(74-551)-prey construct, six out of seven cotransformations resulted in yeast cotransformants that were able to grow on histidine- and adenine-minus plates (Table 1b; columns -T-L-H-A). All these six REPS2 variants have the EH domain in common, indicating that this domain of REPS2 binds to p65. When the REPS2 variants were cotransformed with Clone4 (Table 1c), only the cotransformants with a bait containing the C-terminal part of REPS2 were able to survive the histidine and adenine selection. These results show that the C-terminal part of REPS2 (amino acids 451-659) binds to Clone4, providing a control for specificity of binding of the REPS2 EH domain to p65.

Table 1 The EH domain of REPS2 binds p65(74–263)

	GAL4-DBD	*GAL4-AD*	*Selection −T−L*	*Selection −T−L−H−A*
A	REPS2a(1–659)	Empty prey-vector	+	–
	REPS2a(140–659)	Empty prey-vector	+	–
	REPS2a(1–373)	Empty prey-vector	+	–
	REPS2a(140–373)	Empty prey-vector	+	–
	REPS2a(256–373)	Empty prey-vector	+	–
	REPS2a(256–659)	Empty prey-vector	+	–
	REPS2a(451–659)	Empty prey-vector	+	–
	—	Empty prey-vector	+	–
B	REPS2a(1–659)	p65(74–551)	+	+
	REPS2a(140–659)	p65(74–551)	+	+
	REPS2a(1–373)	p65(74–551)	+	+
	REPS2a(140–373)	p65(74–551)	+	+
	REPS2a(256–373)	p65(74–551)	+	+
	REPS2a(256–659)	p65(74–551)	+	+
	REPS2a(451–659)	p65(74–551)	+	–
	—	p65(74–551)	+	–
C	REPS2a(1–659)	Clone4	+	+
	REPS2a(140–659)	Clone4	+	+
	REPS2a(1–373)	Clone4	+	–
	REPS2a(140–373)	Clone4	+	–
	REPS2a(256–373)	Clone4	+	–
	REPS2a(256–659)	Clone4	+	+
	REPS2a(451–659)	Clone4	+	+
	—	Clone4	+	–
D	REPS2a(1–659)	p65(74–263)	+	+
	REPS2a(140–659)	p65(74–263)	+	+
	REPS2a(1–373)	p65(74–263)	+	+
	REPS2a(140–373)	p65(74–263)	+	+
	REPS2a(256–373)	p65(74–263)	+	+
	REPS2a(256–659)	p65(74–263)	+	+
	REPS2a(451–659)	p65(74–263)	+	–
	—	p65(74–263)	+	–

Yeast AH109 cells were cotransformed with REPS2-bait constructs combined with: (A) empty vector-prey, (B) p65(74–551)-prey, (C) Clone4-prey, (D) p65(74–263)-prey. Equal amounts of the cotransformation mix were plated out on medium that lacked tryptophan and leucine (-T-L; cotransformed cells survive) and on medium that lacked tryptophan, leucine, histidine and adenine (-T-L-H-A; only yeast cells with interaction between bait and prey proteins survive). After 3 days the plates were scored for yeast colonies: (+) = colonies present; (-) = no colonies present.

The EH domain of REPS2 binds the NPF motif region of p65

The next step was to determine which region of p65 interacts with the EH domain of REPS2. An EH domain is a conserved protein-protein interaction domain, first identified in the tyrosine kinase substrate Eps15 (Wong et al., 1995). Different studies showed that EH domain-proteins preferentially bind to peptides or proteins containing an asparagine-proline-phenylalanine (NPF) motif (Fazioli et al., 1993; Paoluzi et al., 1998; Salcini et al., 1997), and such an NPF motif is present in the NF-κB p65 fragment (at amino-acid position 139-141). Alignment of human, mouse and chicken p65 shows that this NPF is conserved (Figure 2a). Furthermore, using structural NF-κB data from the literature, it was shown that the NPF sequence is located in a turn at the surface of the p65 subunit (Figure 2b-d). Similarly, using solution structure data of two other EH-NPF complexes, the NPF was found to be located in a turn when bound into the pocket of the EH domain (de Beer et al., 2000). To collect experimental data to support the hypothesis further, yeast two-hybrid analysis was performed with p65(74-263), a truncated version of p65(74-551), which is missing 288 C-terminal amino-acid residues. It was observed that this truncated p65(74-263), which contains

the NPF sequence, is still able to bind to REPS2 variants that contain the EH domain (Table 1d). This result is in agreement with the hypothesis that the p65-NPF motif is the site that binds to the EH domain of REPS2.

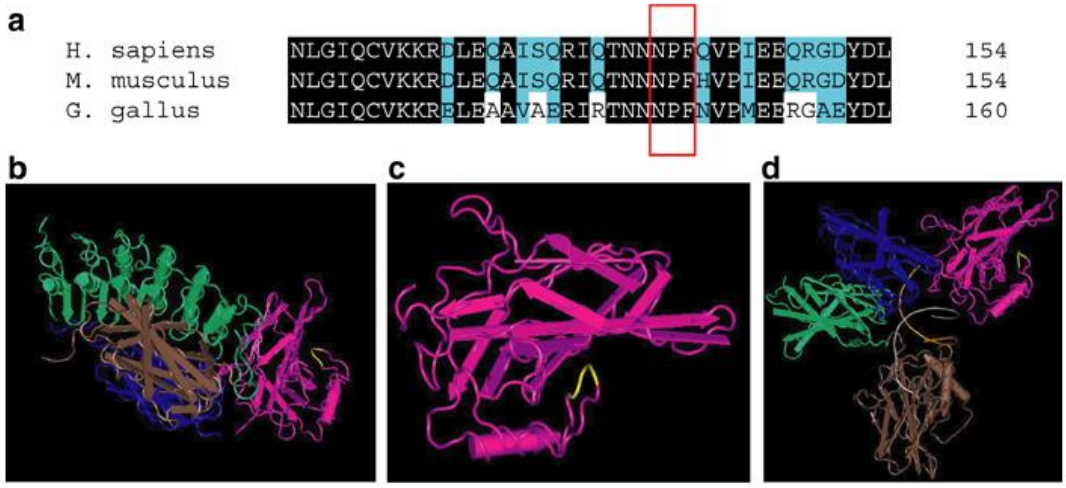

Figure 2 The NPF motif of p65 is present in the human, mouse and chicken proteins, and is located in a turn. (a) The p65 sequence from *Homo sapiens*, *Mus musculus* and *Gallus gallus* were aligned using the DNAMAN program. The red box indicates the NPF motif. (b) Structure of an I-κB/NF-κB complex (Huxford et al., 1998). The complex shown consists of a part of mouse p65 (pink and blue), part of mouse p50 (brown) and part of human I-κB (green). (c) Part of human p65 (pink) subunit from another I-κB/NF-κB complex (Jacobs & Harrison, 1998). (d) Part of mouse p65 (pink and blue) and part of mouse p50 (brown and green) bound to the Ig/HIV-κB DNA site (grey and orange) (Chen et al., 1998a). All three pictures were generated using the Cn3D 3D-structure viewer, which is available at the NCBI website. The NPF amino-acid motif is highlighted in yellow.

Tryptophan 323 in the core of the EH domain is important for REPS2-p65 binding in yeast

In Eps15, a tryptophan residue in the core of the second EH domain is critical for interaction with a peptide containing an NPF motif (de Beer et al., 1998). This interaction was lost when the critical tryptophan residue was replaced by an alanine residue, while substitution by a tyrosine residue affected the binding only slightly (de Beer et al., 1998). The respective tryptophan residue is highly conserved among EH domains, and in REPS2 this residue is located at position 323. To examine whether the conserved tryptophan at position 323 in the EH domain of REPS2 is involved in the binding between REPS2 and p65, this tryptophan was substituted for by either an alanine or a tyrosine residue in REPS2b, and the interaction between these REPS2b mutant proteins and p65 was measured in a quantitative yeast two-hybrid assay. An almost complete loss of reporter signal (beta-galactosidase activity) was observed in yeast cells expressing the REPS2b(W323A)-bait and p65(74-551)-prey, compared to yeast with the wild-type REPS2b-bait and p65(74-551)-prey combination (Figure 3a). This would be in agreement with the observations reported by de Beer et al. (1998).

However, in the present experiments we found that the interaction was also lost for the REPS2b(W323Y)-bait (Figure 3a), which indicates that substitution of the tryptophan 323 residue in the EH domain of REPS2 by either an alanine or a tyrosine residue leads to loss of interaction with NF-κB/p65.
Clone4 binds to the C-terminal region of REPS2b, and it is unlikely that this interaction would be affected by the two point mutations (REPS2b(W323A) and REPS2b(W323Y), respectively). Indeed, wild-type and the two mutant REPS2 baits were found to bind the Clone4-prey construct with similar binding strength, as indicated by a comparable betagalactosidase reporter signal (Figure 3b).
Taken together, the results show that tryptophan 323 in the EH domain of REPS2 is critical for binding of REPS2 to p65 in yeast, lending further support that the EH domain of REPS2 binds the NPF motif(139-141) in p65.

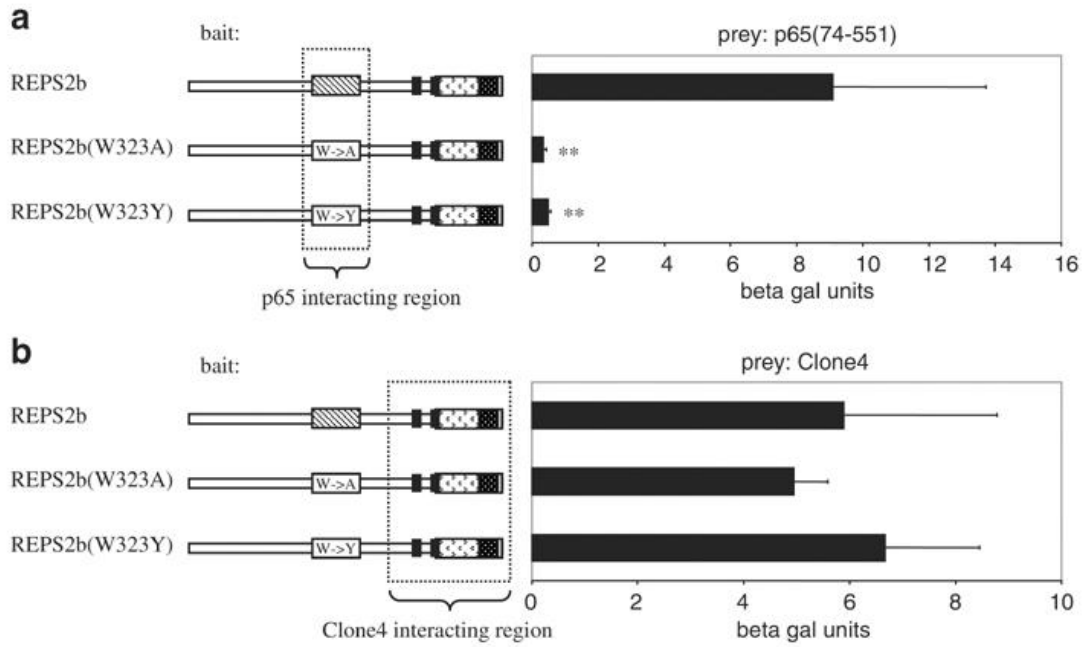

Figure 3 Quantitative two-hybrid assay demonstrating that tryptophan 323 in REPS2 is important for binding to p65. Yeast strain Y190 harboring the REPS2b-, the REPS2b(W323A)- or the REPS2b(W323Y)-bait in combination with the p65(74-551)-prey (a) or the Clone4-prey (b) were cultured. From the cultures, extracts were prepared and β-galactosidase activity was measured. The figure shows the average of three independent experiments for each combination. Differences between wild-type and mutant REPS2b were considered significant (**) at $P<0.01$, as determined by paired Student's *t*-tests.

REPS2 variants that have the EH domain and lack the C-terminal region bind to p65(74-551) in COS-1 cells, but none of the variants bind to p65 in LNCaP cells

From the literature it is known that NF-κB transcription factors are absent in yeast, and bait-prey binding between (putative) NF-κB pathway proteins in yeast, therefore, does not involve endogenous NF-κB pathway regulators. To study the interaction

between REPS2 and p65 in a mammalian environment, a two-hybrid assay was performed in COS-1 cells. Based on the results obtained with the yeast two-hybrid assays (Table 1b), six out of the seven REPS2 variants were expected to bind to p65(74-551). However, using the mammalian two-hybrid assay in COS-1 cells, only three REPS2-bait variants bound to the p65(74-551)-prey, as indicated by the binding signal (Figure 4a; prey: p65(74-551)).

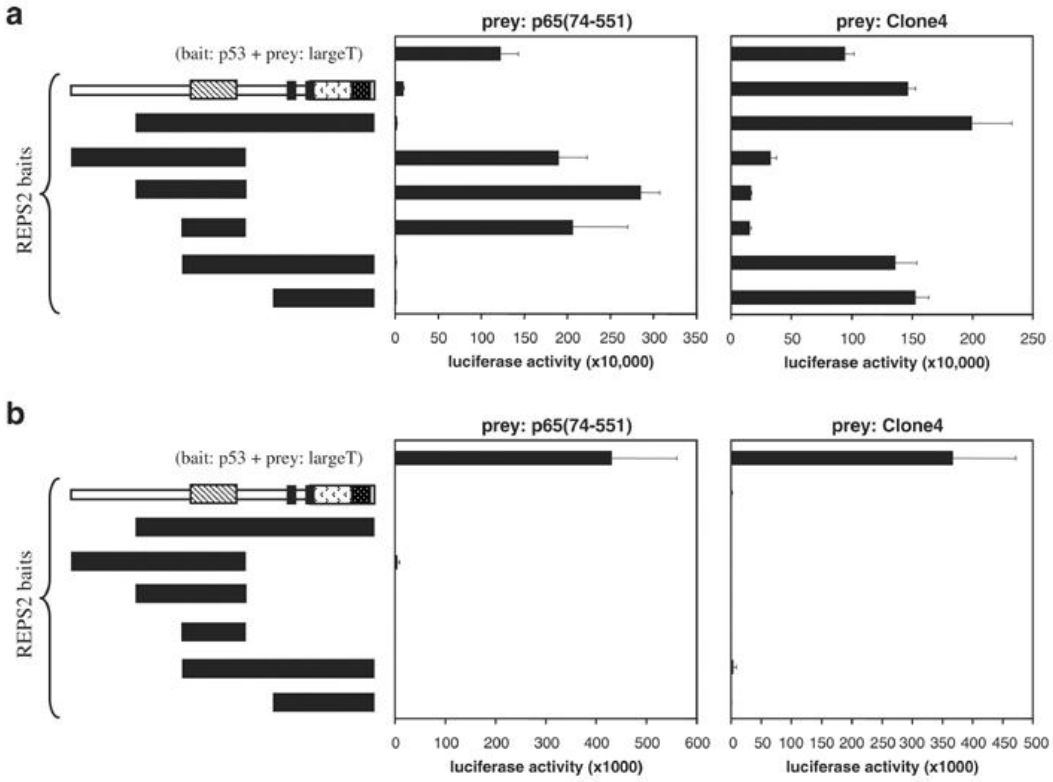

Figure 4 REPS2-p65 and REPS2-Clone4 binding in COS-1 and LNCaP cells. REPS2 bait constructs (left side of the figure) and p65- or Clone4-prey constructs (top of the figures) were cotransfected with the GAL4-luciferase reporter in COS-1 cells (a) or in LNCaP cells (b). Besides these bait and prey constructs, the positive control constructs p53-bait and Large T antigen-prey were also used in this assay. At 48 h after cotransfection, luciferase activity was measured in the cells. For each mammalian two-hybrid assay, two control assays were conducted: empty bait vector+prey and bait+empty prey vector. The control assays generated, compared to the experimental assays, only a low luciferase signal. This experiment has been repeated three times, and the figure shows the data from one representative experiment.

Different factors might be responsible for the failure of several REPS2-baits to generate a binding signal when cotransfected with p65(74-551)-prey: the constructs may not be expressed; the bait-hybrid may not be able to translocate to the nucleus; or REPS2, which is known to induce apoptosis when overexpressed, may also induce apoptosis under these particular circumstances. To exclude these possibilities, REPS2 variants were also cotransfected with Clone4. It was observed that Clone4 bound to all the REPS2 baits that contained the C-terminal region (Figure 4a; prey: Clone4).

These results are in agreement with the data obtained from the yeast two-hybrid assays (Table 1c) and show that all REPS2 hybrid proteins are suitable as bait in the mammalian two-hybrid assays in COS-1 cells.
To study the interaction between REPS2 and p65 in human prostate cells, the mammalian two-hybrid assays were also performed using the androgen-dependent human prostate cancer cell line LNCaP. In contrast to what was observed for COS-1 cells, none of the REPS2-bait variants bound to the p65(74-551)-prey, as indicated by lack of a binding signal (Figure 4b). However, the control binding of REPS2-bait variants to the Clone4-prey also gave negative results (Figure 4b), so that we cannot exclude any possible confounding factors, which may exert an effect in LNCaP cells.

REPS2-p65 and REPS2-Clone4 binding occurs in LNCaP cells after stimulation with PMA

In COS-1 cells the luciferase signals obtained with REPS2 and p65 were higher than the signals produced by positive control binding between p53 and Large T antigen (Figure 4a). In contrast, in LNCaP cells no binding signal for REPS2-p65 or REPS2-Clone4 could be detected, whereas the positive control showed a high binding signal (Figure 4b). A possible explanation for the lack of binding signal in the experimental bait-prey assays in LNCaP cells is that LNCaP cells, compared to COS-1 cells, may contain additional pathways that suppress REPS2-p65 and REPS2-Clone4 interactions. As p65 is a subunit of the NF-κB complex, it was speculated that regulators of the NF-κB pathway could play a role. To test this, mammalian two-hybrid assays were conducted in the presence of the phorbol ester PMA, an activator of the NF-κB pathway. It was observed that for some variants of REPS2, binding to p65 and Clone4 was highly induced by PMA (Figure 5). Expression of REPS2b and p65, and control-binding between p53 and Large T antigen was only marginally affected by PMA, which indicates that the observed effect was not due to increased protein expression, but rather supports the idea that PMA modulates pathways that stimulate binding of REPS2 to p65 and Clone4 in LNCaP cells.

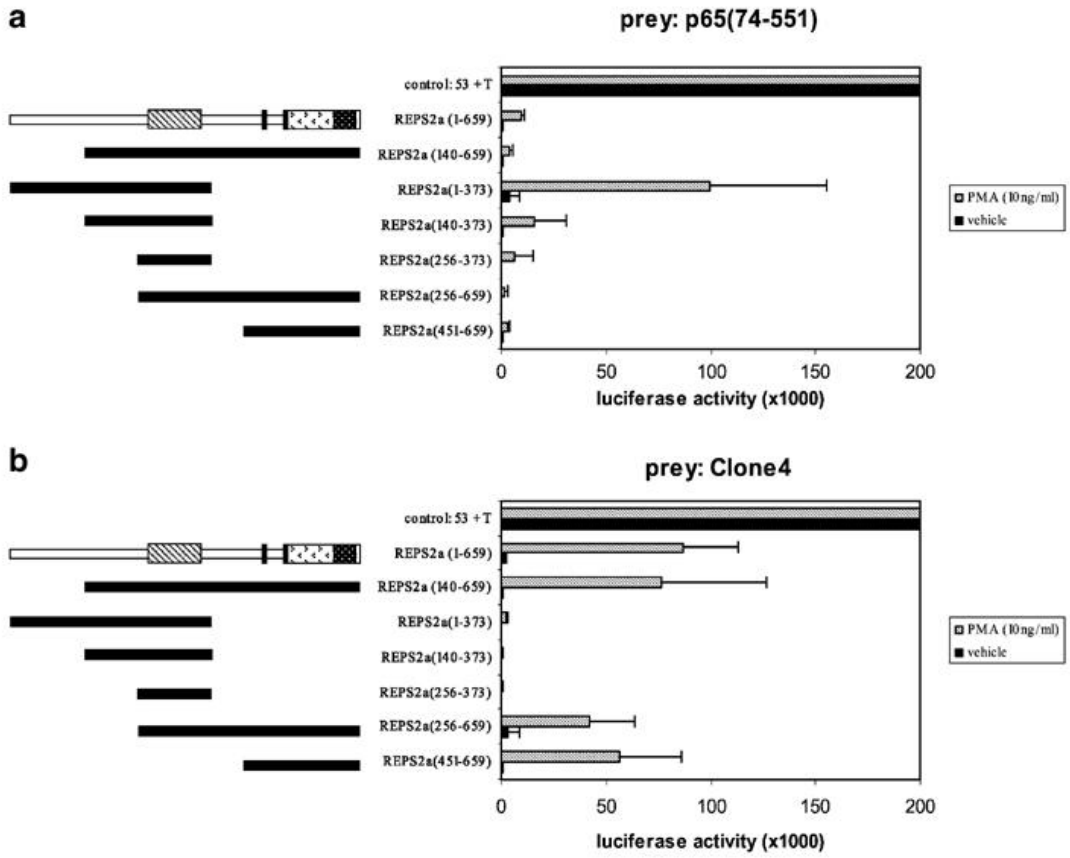

Figure 5 REPS2-p65 and REPS2-Clone4 binding occurs in LNCaP cells after stimulation with PMA. The assays in this figure were performed in the same way as described for Figure 4, except that 24 h after transfection the cells were treated with 10 ng/ml PMA or vehicle.

Tryptophane 323 in the core of the EH domain is important for REPS2-p65 binding in mammalian cells

As shown in Figure 3, in yeast, tryptophan 323 in the EH domain of REPS2(140-659) is important for binding between REPS2(140-659) and p65(74-551). The outcome of similar experiments using mammalian COS-1 and LNCaP cells is shown in Figure 6. As binding between the REPS2(1-373)-bait and the p65-prey produced the highest luciferase signal in LNCaP cells upon treatment with PMA (Figure 5a), this bait was used in the mammalian two-hybrid assays. Figure 6a and b show that substitution of tryptophan 323 to alanine has a dramatic effect on REPS2(1-373)-p65(74-551) binding in COS-1 and LNCaP cells. When tryptophan 323 was substituted for a tyrosine, however, the effect on binding between REPS2(1-373) and p65(74-551) was less severe in both cell lines. These results are in agreement with the results obtained by de Beer et al. (1998) for the binding between a mutated EH domain and an NPF containing peptide (de Beer et al., 1998). It should be noted that the background signals (Figure 6a; panel on the right), which are produced by wild-type REPS2a(1-373) and the two substitution mutated baits, although less high, resembles the signals that are obtained when p65 prey is cotransfected (Figure 6a; panel on the left).

Possibly, there is some binding of an endogenous protein with transactivating activity to the REPS2 baits. In fact, this endogenous protein with transactivating capacity could very well be p65. LNCaP cells probably have less endogenous p65 available for binding to the REPS2 baits (Figure 6b; panel on the right).

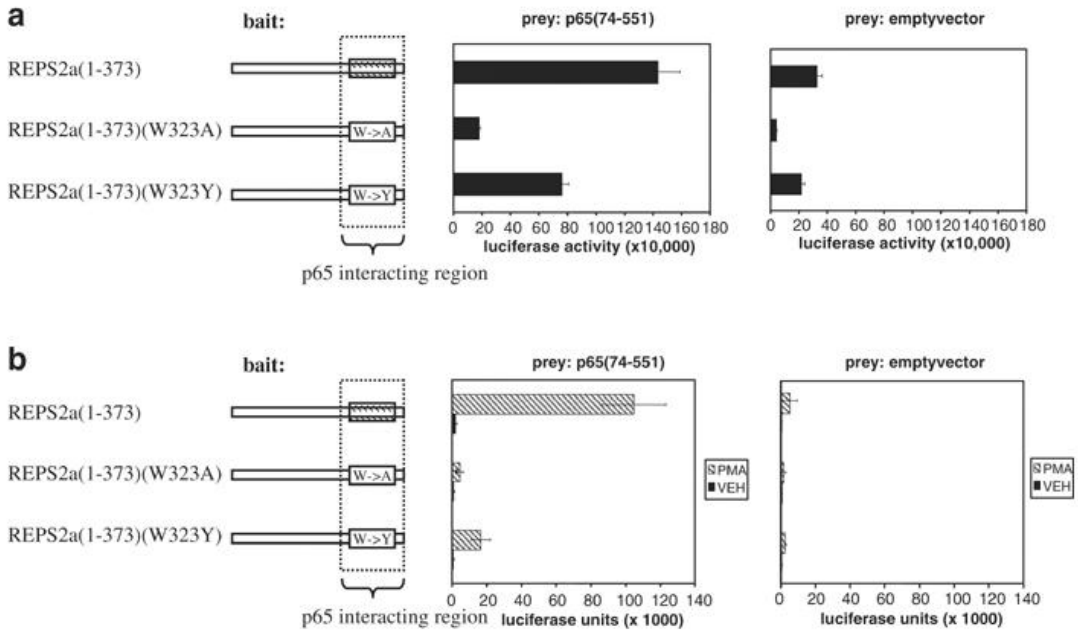

Figure 6 The conserved tryptophan in the EH domain of REPS2 is important for REPS2-p65 binding in mammalian cells. REPS2a(1-373), REPS2a(1-373)(W323A) and REPS2a(1-373)(W323Y) were used as baits in mammalian two-hybrid assays. COS-1 (top) and LNCaP cells (bottom) were cotransfected with reporter, bait and p65(74-551)-prey construct (left side), or the empty-prey vector (right side), as indicated in the figure. (a) COS-1 cells were left untreated for 48 h, and then luciferase activity was measured. (b) LNCaP cells were treated with PMA or vehicle starting at 24 h after transfection, and assayed for luciferase activity 48 h after transfection.

Discussion

Using the yeast two-hybrid system, it was shown that REPS2 was identified as a binding partner of the NF-κB subunit RelA/p65. Furthermore, it was demonstrated that the Eps15 homology domain within REPS2 (EH domain, located at amino acids 265-366) is responsible for this interaction. It has been described that EH domain-proteins preferentially bind to peptides or proteins containing an asparagine-proline-phenylalanine (NPF) motif (Fazioli et al., 1993; Paoluzi et al., 1998; Salcini et al., 1997), and the p65 sequence contains such an NPF sequence at amino acids 139-141. Using crystal structure data from the literature (Chen et al., 1998a; Huxford et al., 1998; Jacobs & Harrison, 1998), it was determined that the NPF in p65 is located at the surface of the protein in a turn of a loop. The position of the NPF in p65 is in agreement with protein-structure data from de Beer et al. (2000), and suggests that the

NPF domain can act as a docking site for proteins like REPS2. Experimental data provided further back-up for this hypothesis: when a large portion of p65 was deleted, the yeast two-hybrid assay still showed normal interaction between the EH domain of REPS2 and p65(74-263). Additional support came when the role of a highly conserved tryptophan in the core of the REPS2 EH domain was studied in quantitative yeast two-hybrid assays. In agreement with De Beer et al. (1998), it was shown that this tryptophan in the EH domain of REPS2 is critical for binding to the NPF-containing protein sequence of p65.

Although the data obtained with the yeast two-hybrid system demonstrated that REPS2 is able to bind to p65, it cannot be concluded that this interaction has a physiological role in the cell. Therefore, the interaction was studied also in mammalian cells, and it was observed that there are significant differences between REPS2-p65 binding in yeast as compared to mammalian cells. First, it was observed that not all REPS2 variants, which - on the basis of the yeast data were thought to interact with p65 - did interact in COS-1 cells. In fact in mammalian cells, the C-terminal part of REPS2 was found to inhibit the interaction between REPS2 and p65. Second, in the human prostate cancer cell line LNCaP, all interactions between EH domain-containing variants of REPS2 and p65 are below detection level. Because p65 is part of the NF-κB complex, it was speculated that NF-κB pathway regulators may suppress REPS2-p65 interaction in LNCaP cells. To modify the activity of NF-κB pathway regulators the assays were conducted in the presence of PMA, which is an activator of the NF-κB pathway. In line with the observations for COS-1 cells, it was observed that in LNCaP cells, in the presence of PMA, significant binding occurred between REPS2 and p65.

As indicated in the Introduction, REPS2 expression is significantly reduced in androgen-independent prostate cancer (Chang et al., 1997; Oosterhoff et al., 2003). Based on these findings, and in view of indications that REPS2 is involved in growth factor signaling (Nakashima et al., 1999), it was hypothesized that REPS2 might play a role in the transition of prostate cancer from androgen-dependent towards androgen-independent growth. Therefore, the newly identified interaction between REPS2 and the NF-κB subunit p65 could also be of importance for our understanding of prostate cancer progression. During prostate cancer progression, and parallel to loss of REPS2 expression, the NF-κB pathway becomes much more active (Chen & Sawyers, 2002; Palayoor et al., 1999). In a developing cancer, activation of the NF-κB pathway would provide the cells with additional cell survival proteins (Sumitomo et al., 1999; Van Antwerp et al., 1996; Wang et al., 1996). For example, Mayo et al. (1997) showed that cells stably transfected with an oncogenic form of Ras die when the NF-κB pathway is blocked. In case of prostate cancer, activation of the NF-κB pathway would imply that the developing cancer becomes much more resistant to removal of androgens (androgen-ablation therapy). Figure 7 shows a sequence of events that may illustrate different and subsequent stages in the development of prostate cancer. Three stages of prostate cancer are depicted. In Figure 7a, an oncogenic signal induces cell growth and cell death pathways in prostate cells. Because, for unknown reasons, the cell death signal is to weak, the prostate cells develop into cancer cells. In Figure 7b, cell death is stimulated because the cancer cells are treated with androgen ablation therapy. The NF-κB pathway cannot prevent this therapy-induced apoptosis, because the pathway is inhibited by REPS2. In Figure 7c, some prostate cancer cells manage to escape cell death with the help of additional cell survival proteins. The observed downregulation of REPS2 during this stage of the disease could be responsible for, or

contribute to, upregulation of cell survival proteins through loss of inhibition of the NF-κB pathway.

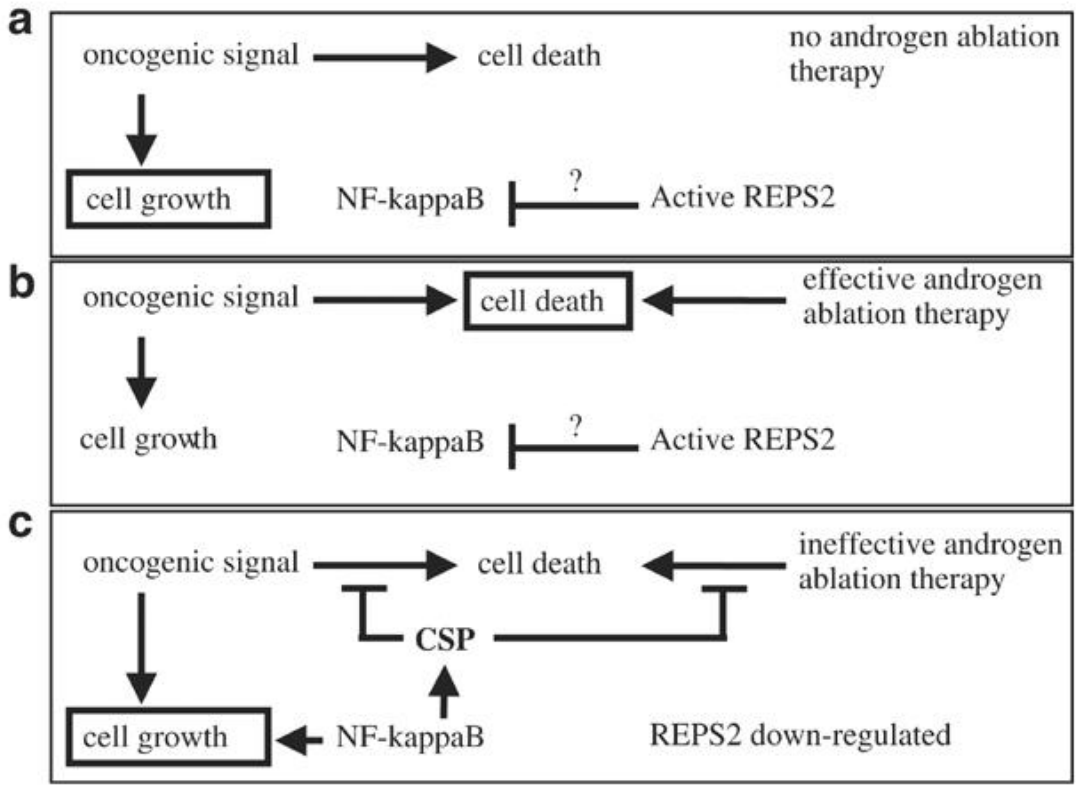

Figure 7 Lack of the REPS2-p65 interaction in relation to prostate cancer. (a, b) Androgen-dependent prostate cancer. The growth signal overrules the endogenous cell death signal (a). However, upon ablation of androgens an additional cell death signal is activated and the cells die of apoptosis (b). In androgen-independent prostate cancer (c), REPS2 expression is relatively low. Lack of REPS2-p65 (NF-κB subunit) interaction is hypothesized to contribute to constitutive activity of the NF-κB pathway. This constitutive activity could then be responsible for expression of extra cell survival proteins (CSP), which would result in survival of prostate cancer cells during androgen ablation.

Many reports in literature describe an antiapoptotic role for the NF-κB pathway; however, there are also some reports that show a proapoptotic role for the NF-κB pathway. In case of prostate cancer for example, Ling et al. (2003) showed that increased NF-κB pathway activity in LNCaP cells promotes cell survival, whereas Kimura & Gelmann (2002) showed a proapoptotic effect of NF-κB signalling in the same cell line. As the NF-κB pathway is highly active in androgen-independent prostate cancer (Chen & Sawyers, 2002; Palayoor et al., 1999), and this activity does not result in cell death, the antiapoptotic effect of NF-κB is depicted in the model (Figure 7). Besides a role in regulating apoptosis, active NF-κB is also able to stimulate proliferation through upregulation of genes that are involved in cell growth (Figure 7c). For example, recently it has been shown that the activated NF-κB

pathway, in androgen-independent prostate cancer cells, is important for upregulation of interleukin-6 (IL-6) (Park et al., 2003; Zerbini et al., 2003). Since IL-6 has also been reported to stimulate growth of prostate cancer cells (Steiner et al., 2003), these cells will become more and more independent of growth factors and androgens.
The molecular mechanism through which downregulation of the REPS2 protein contributes to upregulation of NF-κB activity is not clear at the moment. Because proteins with which REPS2 interacts are all cytoplasmic in localization (Grb2, RalBP1, Epsin, Eps15) and because REPS2 itself was also reported to be located predominantly in the cytoplasm (Oosterhoff et al., 2003), the interaction between REPS2 and p65 probably takes place in the cytoplasm. It can be speculated that REPS2 may inhibit NF-κB activity by retaining p65 in the cytoplasm, but the exact mechanism needs to be studied further.
The NF-κB pathway is constitutively active in many advanced tumours, and is considered a potential target for cancer drug development (Garg & Aggarwal, 2002). Since the mechanisms that are responsible for high NF-κB activity in tumours are not known, studies on the REPS2-p65 interaction may provide additional information with regard to control of activity of the NF-κB pathway during progression of prostate and potentially other cancers.

Materials and methods

Constructs

The complete REPS2a ORF (Oosterhoff et al., 2003) was used as a template to amplify the seven REPS2 variants (Figure 1). In order to facilitate subcloning, an *Eco*RI site was added to the N-terminal primers and a *Sal*I site to the C-terminal primers. The Zero Blunt TOPO PCR Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to clone the PCR products into the pCR-Blunt II-TOPO vector. The REPS2 variants were subsequently subcloned, using the *Eco*RI and *Sal*I sites, into the pGBKT7 (the bait vector from the BD Matchmaker Two-Hybrid System 3, BD Biosciences Clontech, Palo Alto, CA, USA) and into the pM vector (the bait vector from BD Matchmaker Mammalian Two-Hybrid Assay Kit, BD Biosciences Clontech). All seven REPS2 variants have been sequenced. The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to construct the REPS2 fragments containing the W323A and W323Y pointmutations. The isolated yeast two-hybrid prey plasmids pACT2clone4 (unknown hypothetical protein) and the pACT2clone8 (encodes for p65(74-551)) were used as template to PCR the Clone4 fragment and the p65 fragment. Again, to facilitate subcloning an *Eco*RI site was added to the N-terminal primer for the Clone4 fragment and a *Sal*I site to the C-terminal primer. For the p65 fragment N-terminal a *Bam*HI site was introduced and C-terminal a *Hind*III site. The PCR fragments were cloned into the pCR-Blunt II-TOPO vector and subsequently subcloned into the pVP16 vector (the prey vector from the Mammalian two-hybrid system, BD Biosciences Clontech). DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) and the DNA Gel Extraction Kit from Millipore (Millipore Corporation, Bedford, MA, USA) was used to isolate fragments from gel. The Rapid DNA Ligation Kit (Roche, Mannheim, Germany) was used for the ligation reactions.

Yeast two-hybrid screen

The BD Matchmaker Two-Hybrid System 3 (BD Biosciences Clontech) was used for the yeast two-hybrid screen. The pGBKT7-REPS2a bait construct was introduced into the *S. cerevisiae* AH109 strain and protein expression was verified on Western blot (not shown). The AH109 strain with the REPS2a bait was used to screen the Human Prostate Matchmaker cDNA Library (BD Biosciences Clontech). The screening was performed as described in the BD Matchmaker Two-Hybrid System 3 manual, with the exception that carrier DNA from Salmon Testes was used (Sigma-Aldrich Corporation, St Louis, MI, USA). From yeast cells that survived binding selection the prey plasmid was rescued according to the protocol described by Hoffman & Winston (1987).

Yeast two-hybrid assays

The *S. cerevisiae* AH109 strain was used in the qualitative yeast two-hybrid assays as shown in Table 1. The AH109 yeast strain was cotransformed, with the appropriate bait and prey vectors, according the Quick and Easy TRAFO Protocol (Gietz & Woods, 2002). Since the AH109 strain has low expression of the *lac*Z reporter gene the *S. cerevisiae* Y190 strain, which has high expression of the *lac*Z gene, was used in the quantitative yeast two-hybrid assays as shown in Figure 3. A liquid culture assay, which used ONPG as substrate (Yeast Protocols Handbook; BD Biosciences Clontech manual PT3024-1), was used to measure the activity of the *lac*Z reporter. Because the Y190 is relatively difficult to cotransform the Y190 strain was first transformed with the appropriate prey vector and then the appropriate bait vector was introduced.

Mammalian two-hybrid assays

The BD Matchmaker Mammalian Two-Hybrid Assay Kit (BD Biosciences Clontech) was used for the mammalian two-hybrid assays. Instead of the pSG5CAT reporter vector, which is the reporter from the Mammalian Two-Hybrid Assay Kit, a 5 ´ GAL4 binding site-luciferase reporter was used. COS-1 and LNCaP cells were cotransfected using Fugene 6 Transfection Reagent (Roche, Indianapolis, IN, USA) with 200 ng DNA mix (80 ng bait, 80 ng prey and 40 ng reporter) per well of a 24 wells plate. PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich Corporation) was dissolved in 100% ethanol and used in a 10 ng/ml concentration. COS-1 cells were maintained in DMEM/F12 containing 5% dextran-coated charcoal treated fetal calf serum (DCC-FCS). LNCaP were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) and were used between passages 27 and 37. Both cell lines were cultured at 37°C in a 5% CO2 atmosphere and standard antibiotics were added to the media. Luciferase activity was determined 48 h after the start of transfection with the Topcount NXT^{TM} microplate luminescence counter (Packard Bioscience BV; Meriden, CT, USA). Cells were lysed with a standard luciferase lysis buffer and subsequently a part of the lysate was transferred to the Topcount 96 wells microplate. Just prior to the measurement, Steady Glo substrate (Promega, Madison, WI, USA) dissolved in Steady Glo buffer was added to the wells.

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)

References

References are listed on pages 88-93.

CHAPTER 4

Additional data on REPS2-p65 interaction and preliminary data on the interaction of REPS2 with several other human proteins

Additional data on REPS2-p65 interaction and preliminary data on the interaction of REPS2 with several other human proteins

4.1 Introduction

The yeast two-hybrid screen with REPS2 as bait resulted in the identification of several human proteins that seem to be able to physically bind to REPS2 (Appendix). In Chapter 3 the protein-protein interaction between REPS2 and the NF-κB subunit p65 is described. In this Chapter the NF-κB pathway and the possible consequence that loss of REPS2-p65 interaction has on the activity of this pathway in prostate cancer cells is examined. Furthermore extended data on REPS2-p65 interaction is shown. In the second part of this Chapter the interaction between REPS2 and several other human proteins that were identified as REPS2 binding partner (Appendix) is described. Using full length and truncated REPS2, most of the identified interactions during the yeast two-hybrid screen could be confirmed. These experiments also provided information on the location of the binding site in REPS2 for several of the proteins. Two of the identified proteins, STAT6 and TRAF4 were studied in more detail.

4.2 The NF-κB pathway and REPS2-p65 interaction

REPS2 might be a regulator of the NF-κB pathway since the REPS2 protein interacts with the NF-κB subunit p65 (Chapter 3). NF-κB (nuclear factor κ chain transcription in B cells) was discovered in biochemical experiments on the basis of its requirement in B cells for transcription of the gene encoding κ light chain of immunoglobulins (Ghosh et al., 1998; Sen & Baltimore, 1986). NF-κB is a dimer of two REL proteins (also called NF-κB subunits). There are 5 REL proteins, RELA which is the p65 subunit, RELB, c-REL, p50 and p52. All REL proteins have a DNA binding domain and a dimerization domain, but only RELA, RELB, and c-REL have a transactivation domain. Through a dimerization domain, REL proteins form homodimers and/or heterodimers. The p65 NF-κB subunit can form homodimers, and heterodimers with c-REL or p50. The p50/p65 heterodimer is the most abundant form of NF-κB. Specific combinations of REL subunits can precisely regulate the activity of different genes (Chen & Greene, 2004).
In resting cells, the NF-κB hetero- (or homo-) dimer is retained in the cytoplasm by direct binding to the inhibitor IκB. Binding of IκB to the dimer masks the nuclear localisation signals in the subunits (Hayden & Ghosh, 2004; Karin, 1999). In response to an extracellular signal or factor that can activate the pathway, IκB is phosphorylated at two N-terminal serine residues by the IκB kinase (IKK) complex, which consists of the two kinases CHUCK and IKBKB, and the regulatory subunit IKBKG. Phosphorylated IκB is targeted for ubiquitination, and subsequent degraded by the proteasome (Hayden & Ghosh, 2004; Karin, 1999). The release of IκB from the

NF-κB dimer exposes the nuclear localisation signals in the subunits, which results in translocation of the dimer to the nucleus. In the nucleus, the dimer binds to DNA at specific κB-binding sequences, and regulates transcription of different target genes. One of the target genes is the gene encoding the inhibitor IκB. Newly synthesized IκB can enter the nucleus, pull NF-κB off DNA, and export NF-κB back to its resting state in the cytoplasm, which implies a negative feedback in the NF-κB pathway (Chiao et al., 1994; Gilmore, 1999) (Figure 1).

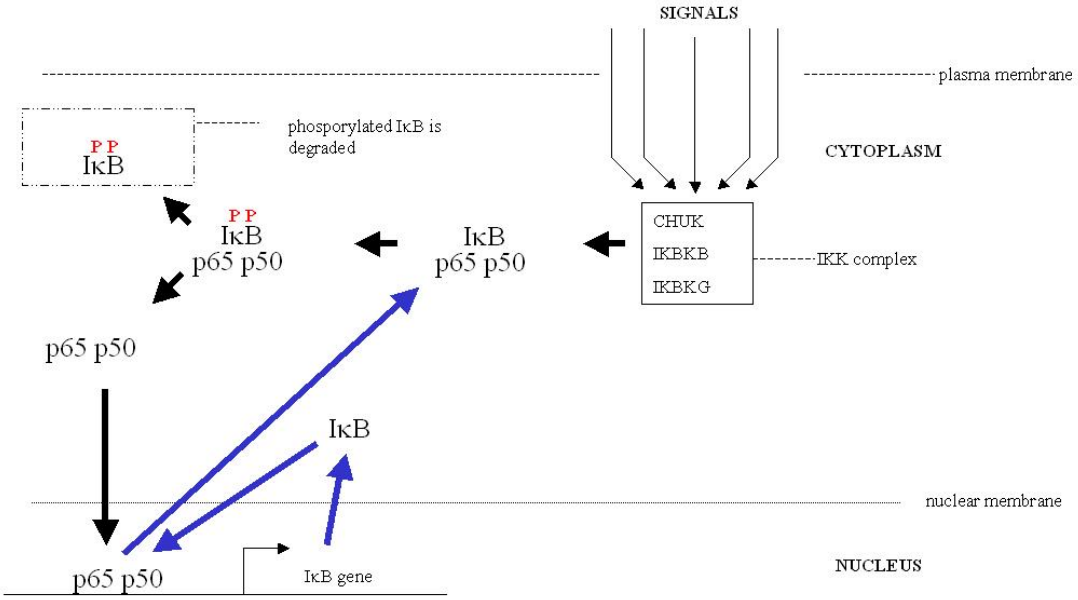


Figure 1 NF-κB signal transduction
For explanation see Paragraph 4.2. Black lines indicate the activating pathway; blue lines indicate the inactivating pathway; the red Ps signify phosphorylation.

In addition to the *IκB* gene, the NF-κB dimer has many other target genes, of which the gene products are involved in a wide variety of cellular processes (Mercurio & Manning, 1999; Pahl, 1999). In 1996, reports from three separate studies demonstrated that NF-κB is able to promote cell survival (Beg & Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996), showing an important role for NF-κB in the life-death balance of cells (Barinaga, 1996). Consequently, the NF-κB pathway might be implicated also in several steps in cancer development and progression. A disturbed and constitutively active NF-κB pathway may suppress endogenous and/or exogenous apoptosis signals by promoting cell survival, and this may lead to cancer progression. NF-κB is activated in several cancers (Garg & Aggarwal, 2002), including pancreatic cancer (Algul et al., 2002; Sclabas et al., 2003), breast cancer (Cao & Karin, 2003; Nakshatri et al., 1997), and in response to chemotherapy and radiation (Orlowski & Baldwin, 2002).

The NF-κB pathway in prostate cancer cells

In prostate cancer, it has been reported that the NF-κB pathway is constitutively active in AIPC but not in ADPC (Chen & Sawyers, 2002; Gasparian et al., 2002; Palayoor et al., 1999). In agreement with literature, we found, using a NF-κB–luciferase reporter, that the AIPC cell lines DU145 and PC3 have and the ADPC cell line LNCaP does not have a constitutively active NF-κB pathway (data not shown). The present results (Chapter 3) indicate that REPS2 interacts with the NF-κB subunit p65, which may provide an inhibitory control of NF-κB signalling. The high activity of the NF-κB pathway in AIPC is associated with a low level of REPS2 protein (see Chapter 2). Therefore, the decreased REPS2 protein level in AIPC (compared to ADPC) might result in an increased activity of the NF-κB pathway in AIPC.

Can overexpression of REPS2 inhibit the NF-κB pathway?

If a decrease in REPS2 protein level indeed contributes to an increase in the activity of the NF-κB pathway, then overexpression of REPS2 in an AIPC cell may negatively effect the constitutive activity of the NF-κB pathway. We tested this in TSU cells, which is a cell line that was first considered as an AIPC cell line; however, now the cell line appears to be from bladder origin (van Bokhoven et al., 2001). TSU was cotransfected with either a NF-κB reporter and a REPS2 expression vector, or a NF-κB reporter and an empty expression vector. REPS2 expression strongly decreased the NF-κB reporter activity, which suggests that overexpression of REPS2 inhibits the constitutively active NF-κB pathway in the TSU cell line (Figure 2 lane 1 versus lane 2). Figure 2 lane 1 versus lane 5 shows that the TSU cell line has a constitutively active NF-κB pathway, since the NF-κB reporter produced a high luciferase signal and the mutant NF-κB reporter did not.

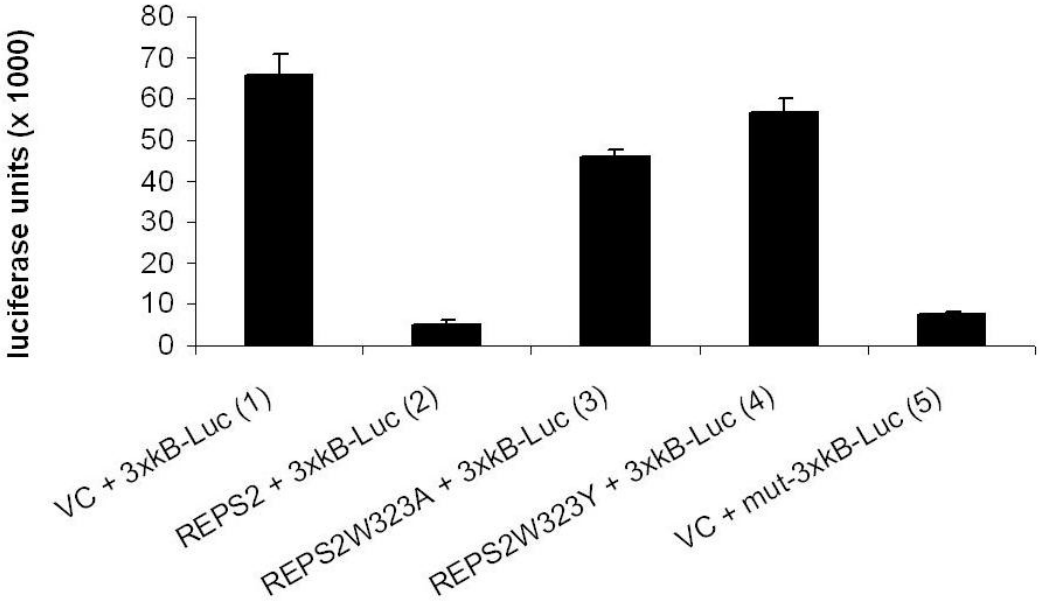


Figure 2 Effect of REPS2 on the NF-κB pathway in TSU cells. TSU cells were cotransfected with expression vectors and reporters as mentioned in the Figure. Luciferase activity was measured 41 hours after transfection. VC: empty vector; mut-3xkB-Luc: mutant NF-κB reporter.

A tryptophan on position 323 in the REPS2 protein seems to be essential for good binding to p65 (Chapter 3). Two REPS2 constructs with point mutations on this position were also used in the experiment. Interestingly, in the experiment these two point-mutated REPS2 variants did not inhibit the constitutively active NF-κB pathway in the TSU cell line as wild type REPS2 did (Figure 2 lane 2 versus lane 3 and 4). Unfortunately, for unknown reasons this experiment could not successfully be repeated (in subsequent experiments, overexpression of wild type REPS2 did not effect the activity of the NF-κB pathway in TSU cells). We also could not demonstrate an inhibiting activity of REPS2 towards the constitutively active NF-κB pathways in the AIPC cell lines DU145 and PC3. Inhibition by overexpression of REPS2 of a TNFα- or PMA- activated NF-κB pathway in ADPC LNCaP cells could not be demonstrated either. Taken together, the results suggest that the inhibiting activity of REPS2 towards the NF-κB pathway is very sensitive and might be influenced by the activity of several upstream signal transduction pathways (see the part below and see the General Discussion and Future Directions in Chapter 5).

Signal transduction pathways and REPS2 involved protein-protein interaction

Exposure of a cell to a signal transduction pathway activator can induce changes in conformation and activity of specific membrane-bound and/or intracellular proteins, which subsequently targets downstream proteins. Affected proteins may transiently change their binding activity towards different protein partners. For example, co-immunoprecipitation experiments showed that activation of TNF receptor induced formation of TRAF4/p70S6K complexes (Fleckenstein et al., 2003). Changes in protein-protein binding affinity may be a consequence of phosphorylation of the protein(s), or a change in protein conformation, or a combination of modifications. When REPS2 is subject to such modifications, this may change binding strength towards specific binding partners.

NF-κB is induced by over 150 stimuli (Pahl, 1999). Exposure of cells to such a stimulus leads to activation of the NF-κB pathway, mostly through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (Figure 1). The key regulatory step in this pathway involves activation of the IκB kinase (IKK) complex (Hayden & Ghosh, 2004; Karin, 1999) (Figure 1). Each NF-κB pathway activator induces, besides the pathway that leads to IKK activation, specific additional signal transduction pathways that may also impact on NF-κB activity.

PMA and TNFα, which are both activators of the NF-κB pathway, use different endogenous proteins to activate NF-κB. In U2OS cells for example activation of the IKK complex in response to PMA is mediated by PKC-alpha, since both the PKC inhibitor GF109203 and a catalytically inactive PKC-alpha mutant inhibit activation of endogenous IKK by PMA, but not by TNFα (Vertegaal et al., 2000).

EGF is a well-known NF-κB pathway activator (Biswas et al., 2000). REPS2 is thought to play a role downstream of the EGF receptor (Chapter 1). Therefore, the identified REPS2-p65 interaction might specifically impact on EGF-induced NF-κB activation. REPS2 may for example serve as a scaffold protein for p65, pulling p65 into a complex with other proteins that may act on p65 to modify its activity in the NF-κB signalling pathway.

In Chapter 3, the androgen-dependent human prostate cancer cell line LNCaP was used as host for REPS2-p65 mammalian two-hybrid assays to study the interaction between REPS2 and p65. However, these assays did not result in REPS2-p65 binding signals (Chapter 3, Figure 4b). LNCaP cells have a low transfection efficiency, but this does not explain the negative result, since the p53-largeT positive control assay showed a binding signal (Chapter 3, Figure 4b). We suggested that pathways might be present in LNCaP cells that suppress REPS2-p65 binding and, because p65 is a subunit of the NF-κB complex, regulators of the NF-κB pathway could play a role. Using NF-κB pathway activators, we might be able to modulate the activity, or the downstream effect, of these regulators and binding between REPS2 and p65. Indeed, it was found that exposure of the cells to phorbol 12-myristate 13-acetate (PMA), a well-known activator of the NF-κB pathway, resulted in activation of interaction between REPS2 and p65 (Chapter 3, Figure 5a). This result has led to the following questions: (1) do other activators of the NF-κB pathway, for example TNFα, affect signalling pathways that influence REPS2-p65 interaction in LNCaP cells; and (2) do PMA sensitive signalling pathways affect other interactions in which REPS2 is involved, for example the REPS2-TRAF4 binding? The first question is addressed below, and the second question is addressed in Paragraph 4.3.

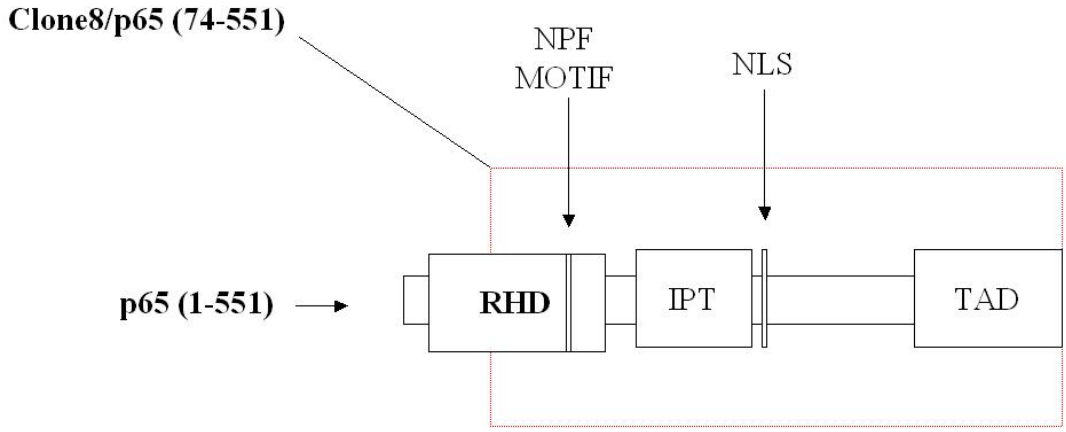


Figure 3 Schematic representation of the NF-κB subunit p65
For explanation of the depicted domains and motif, see text in Paragraph 4.2.
The prey amino acid sequence p65 (74-551) (See Appendix Table 1 Clone8) is indicated with a red box. Note: The RHD and IPT domains that are depicted in the schematic representations were determined with the use of the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2003). The positions of the NLS and the TAD in p65 were deduced from a schematic representation of p65 in literature (Masui et al., 2002).

Schematic representation of the NF-κB subunit p65

The NF-κB p65 subunit, RELA, is a protein of 551 amino acid residues. In the N-terminal half of the protein, the REL homology domain (RHD) is located, which is

involved in DNA binding. In the middle part of the protein, an IPT (immunoglobulin-like fold, plexins, transcription factors) domain is located, which is involved in dimerization with other subunits of the NF-κB-family and in binding to IκB. The p65 subunit also contains a nuclear localization signal (NLS), and a transactivation domain (TAD) is located near the C-terminus (Figure 3). The NPF motif that is depicted in this schematic representation of p65 is discussed in Chapter 3.

REPS2 binding to p65 in LNCaP cells upon stimulation with TNFα

To see if TNFα responsive pathways are able to affect the binding between REPS2 and p65 in LNCaP cells, mammalian two-hybrid assays with REPS2 (140-659) as bait and p65 (74-551) as prey were conducted. The preliminary results showed that incubation of the cells with TNFα results in a stimulatory effect on binding between REPS2 and p65 (Figure 4 lane 1 versus lane 2).

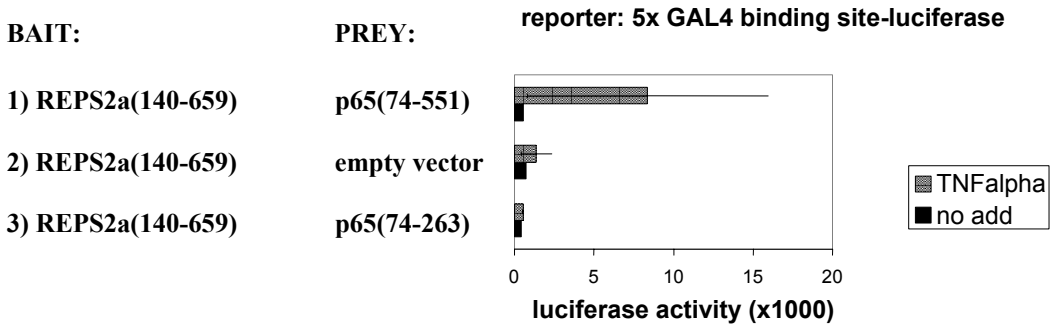


Figure 4 The effect of TNFα responsive pathways on REPS2-p65 interaction in LNCaP cells
The assays were conducted as described in Chapter 3 Figure5.

REPS2a (140-659) binds to p65 (74-551) but not to C-terminal truncated p65 (74-263) in LNCaP cells upon stimulation with TNFα

In Chapter 3, it is shown that REPS2 variants that contain the EH domain bind to p65 (74-263) in yeast-two hybrid assays (Chapter 3, Table 1D). The p65 (74-263) prey is a C-terminally truncated version of p65 (74-551), but it still contains the NPF motif that is the candidate site to bind into the EH domain of REPS2 (see Chapter 3). The p65 (74-263) fragment was subcloned into the mammalian two-hybrid prey vector to study the effect of this C-terminal truncation on REPS2-p65 binding in mammalian cells. The mammalian two-hybrid assay was conducted with REPS2 (140-659) as a bait and p65 (74-263) as a prey, in LNCaP cells in the presence or absence of TNFα. It was found that REPS2 (140-659) does not bind to p65 (74-263) in the presence of TNFα, in contrast with binding to p65 (74-551) (Figure 4, lane 3 versus lane 1). This observation suggests that the p65 (264-551) C-terminal amino acid sequence may contain regions, which exert a regulatory function in the series of events leading to interaction between REPS2 and p65 in cells that are activated by TNFα signalling.

4.3 Interaction of REPS2 with several other human proteins

In the yeast two-hybrid screen (see Appendix) we found, besides the interaction of REPS2 to p65 and Clone4 (see Chapter 3 and this Paragraph 4.2), several other preys that bound to REPS2 bait (Appendix, Table 1). To confirm physical interaction between REPS2 and several of these identified proteins, additional yeast two-hybrid assays were conducted.

Additional yeast two-hybrid assays

Yeast cells which show activation of the adenine selection marker, by gain of the ability to produce adenine, are considered as a positive clone (Appendix, Table 1). Not in al positive clones bait-prey interaction may occur. A useful control to exclude false positives is to perform the assay with a bait hybrid that carries no, or a mutated, bait amino acid sequence. Thirteen clones were analysed using such a control, and Table 1 (NO BAIT versus REPS2a (1-659)) shows that only Clone3 survived adenine selection without the REPS2a amino acid sequence present in the bait hybrid. All the other clones failed to survive the adenine growth selection when the REPS2a sequence was absent, but managed to survive the selection when the REPS2a sequence was present, which indicates physical binding between bait and prey. Possibly, the Clone3 amino acid sequence binds the GAL4 binding sites in the promoter region upstream of the adenine marker, or directly binds to the GAL4-DBD, resulting in a false positive signal.
When in the yeast two-hybrid assay (or screen) “the prey bites the bait”, the GAL4 transcription factor is reconstituted and can activate transcription of the genes encoding the selection markers (Appendix, Figure 1b). This reconstitution of GAL4 does not require full-length bait proteins, but can also be obtained for isolated domains of the bait protein. Two partial REPS2 amino acid sequences were used as bait in the yeast two-hybrid assay: REPS2a (256-659) and REPS2a (451-659), which represent truncated forms of the REPS2a bait. These experiments aimed to get information about the location of the site(s) where the prey amino acid sequences are interacting with REPS2. This is described in more detail in Chapter 3. Table 1 summarizes the data. For example, it is shown that the prey Clone4 binds to all the REPS2 bait amino acid sequences that were used in the present assays. The results suggest that Clone4 binds to the C-terminal part of REPS2. In contrast, Clone2 does not bind to REPS2a (256-659) and REPS2a (451-659), which suggests that Clone2 binds to the N-terminal region of the REPS2 protein.
In the paragraphs below, we describe and discuss several aspects of the interactions of REPS2 with prey sequences representing TRAF4 and STAT6. These two proteins seemed interesting candidate REPS2 binding partners for further study, because of their well-known function in signal transduction. Moreover, these two proteins are functionally connected to some parts of the NF-κB pathway. The preys that were used are not the full-length proteins. Rather, we have used relevant functional domains of TRAF4 and STAT6.

4.4 TNF receptor-associated factor 4 (TRAF4)

TRAF4 is a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family. Six members of the TRAF family have been identified. TRAFs can function as adaptor proteins that couple certain membrane-bound receptors, including the TNF receptor to signalling pathways. TRAF proteins are thought to be important regulators of cell death and cellular responses to stress (Bradley & Pober, 2001; Wajant et al., 2001). For example, it has been demonstrated that TRAF4 can inhibit FAS-induced apoptosis (Fleckenstein et al., 2003).
An activated TNF receptor can stimulate the NF-κB pathway, and therefore it is not surprising that roles for TRAFs in regulation of the NF-κB pathway have been reported. TRAF2 (Rothe et al., 1995), TRAF5 (Nakano et al., 1996), and TRAF6 (Cao et al., 1996; Ishida et al., 1996) can activate the NF-κB pathway, whereas for TRAF4 a role in inhibition of the NF-κB pathway has been described (Ye et al., 1999). Furthermore, it has been demonstrated that the NF-κB pathway can regulate expression of the *TRAF4* gene. In primary T-cells and Jurkat cells stimulated with the NF-κB inducers TNFα or phorbol 12-myristate 13-acetate (PMA), TRAF4 mRNA expression was found to be rapidly upregulated (Glauner et al., 2002).

Table 1 Yeast two-hybrid assays

	BAIT:	NO BAIT	REPS2a (1-659)	REPS2a (256-659)	REPS2a (451-659)
PREY:					
Clone 2		-	+	-	-
Clone 3		+	+	+	+
Clone 4		-	+	+	+
Clone 6		-	+	+	-
Clone 7		-	+	-	-
Clone 8	(p65)	-	+	+	-
Clone 10	(STAT6)	-	+	+	-
Clone 20.3		-	+	-	-
Clone 23		-	+	+	-
Clone 28		-	+	+	-
Clone 38		-	+	+	-
Clone 41		-	+	-	-
Clone 44	(TRAF4)	-	+	+	+

The *S. cerevisiae* AH109 yeast strain was cotransformed with the appropriate bait and prey vectors, according the Quick and Easy TRAFO Protocol (Gietz & Woods, 2002). The cotransformation mix was plated out on medium that lacked tryptophan, leucine, histidine and adenine. Only yeast cells with interaction between bait and prey proteins activate the adenine marker and will therefore survive. After 3 days, the plates were scored for yeast colonies: (+) colonies present; (-) no colonies present. See Appendix Table 1 for a description of the clones and Figure 5 for schematic representations of the baits.

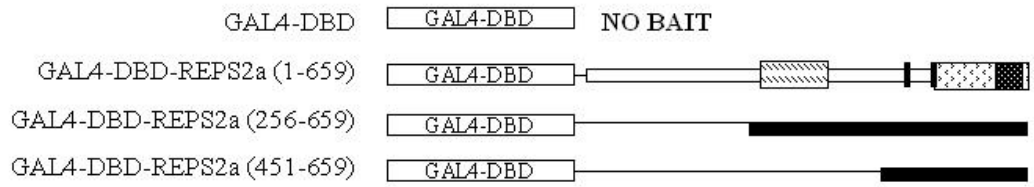


Figure 5 Baits used in the yeast two-hybrid assays
Schematic representations of the four baits that were used in the yeast two-hybrid assays that are described in Table 1.

Schematic representation of the TRAF4

TRAF4 is a protein consisting of 470 amino acid residues. The protein contains: a RING-finger (really interesting new gene) domain; three zf-TRAF (TRAF-type zinc finger) regions; a coiled-coil domain; and a TRAF domain (Figure 6). A RING-finger domain is a specialized type of Zn-finger of 40 to 60 amino acid residues that binds two zinc atoms and is probably involved in protein-protein interactions. The three zf-TRAF regions and the coiled-coil are probably also involved in protein-protein interaction. The TRAF domain, which is present in all TRAFs, is important for binding to the cytoplasmic domain of different plasma membrane receptors, and to other TRAF proteins.

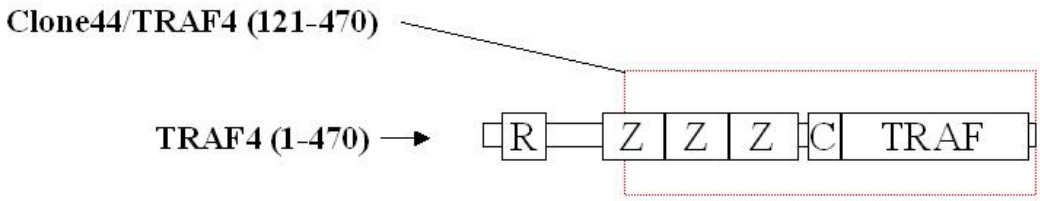


Figure 6 Schematic representation of TRAF4
For explanation of the depicted domains, see text in Paragraph 4.4.
The prey amino acid sequence TRAF4 (121-470) (See Appendix Table 1 Clone 44) is indicated by a red box. R = RING domain, Z = zf-TRAF domain, C = coiled-coil domain.
Note: The domains that are depicted in the schematic representations were determined with the use of the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2003). The putative coiled-coil in TRAF4 was determined by an algorithm described in literature (Lupas et al., 1991).

TRAF4 binds to the C-terminal region of REPS2

The Clone44 prey amino acid sequence is an N-terminal truncated form of TRAF4, TRAF4 (121-470) that lacks the RING-finger domain and part of the first Zn-finger domain. TRAF4 (121-470) binds to all the REPS2 bait sequences that were used in

the assays; as a control, there was no binding to the DBD (DNA-binding domain) of GAL4 (Table 1). This indicates that TRAF4 (121-470) binds to a site in REPS2 that is located somewhere in the C-terminal region of REPS2. In that region of REPS2, three proline-rich motifs (PRMs) and a coiled-coil are located (Chapter 1, Figure 1). It is not likely that TRAF4 (121-470) binds to the PRMs in REPS2, since the TRAF4 (121-470) amino acid sequence does not contain a SH3 domain (Figure 6), which is a protein-protein interaction domain that binds to PRMs (Chapter 1, Paragraph 1.4). The coiled-coil in the TRAF4 (121-470) amino acid sequence (Figure 6), however, might bind to the coiled-coil in REPS2. This can be studied further in the yeast two-hybrid assay using additional truncation mutants of REPS2 and TRAF4.

REPS2a (140-659) does not bind strongly to TRAF4 (121-470) in COS-1 cells

Interaction between REPS2 and TRAF4 was studied in COS-1 cells. In the experiment that is shown in Figure 7, the interaction between REPS2 (256-373) and p65 (74-551) was used as a positive control, since this interaction produced a high luciferase-binding signal (see Chapter 3, Figure 4a). The mammalian two-hybrid assay with REPS2a (140-659) as bait and TRAF4 (121-470) as prey showed a weak interaction in COS-1 cells (Figure 7, lane5). In contrast, the positive control binding showed a strong interaction (Figure 7, lane 2). Whether other (truncated) REPS2 baits bind stronger to TRAF4 (121-470) in COS-1 cells remains to be studied, however, the next paragraph shows that PMA sensitive signalling pathways induce binding between REPS2a (140-659) and TRAF4 (121-470) in LNCaP cells.

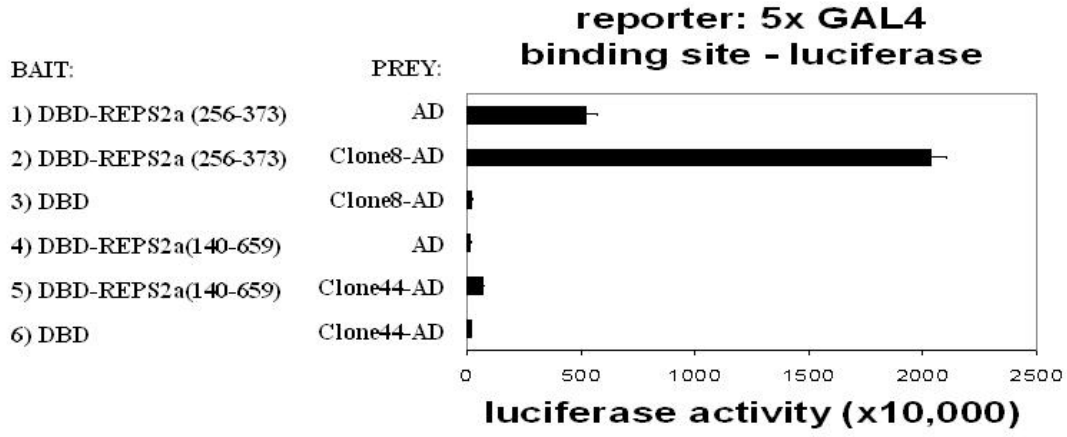


Figure 7 REPS2-TRAF4 interaction in COS-1 cells
Clone44 is a code name for TRAF4 (121-470) (see Appendix). The assays were conducted as described in Chapter 3 Figure 4.

REPS2 binding to TRAF4 in LNCaP cells upon stimulation with PMA

To study a possible effect of PMA-activated signalling on REPS2-TRAF4 binding in LNCaP cells, the mammalian two-hybrid assay with REPS2a (140-659) as bait and

TRAF4 (121-470) as prey was conducted in LNCaP cells incubated in the presence or absence of PMA. It was found that exposure of the cells to PMA results in marked stimulation of the binding between REPS2 (140-659) and TRAF4 (121-470) (Figure 8, lane 3). PMA had only a marginal effect on the positive control binding between p53 and largeT (Figure 8, lane 1), which shows specificity of PMA towards the REPS2-TRAF4 binding.

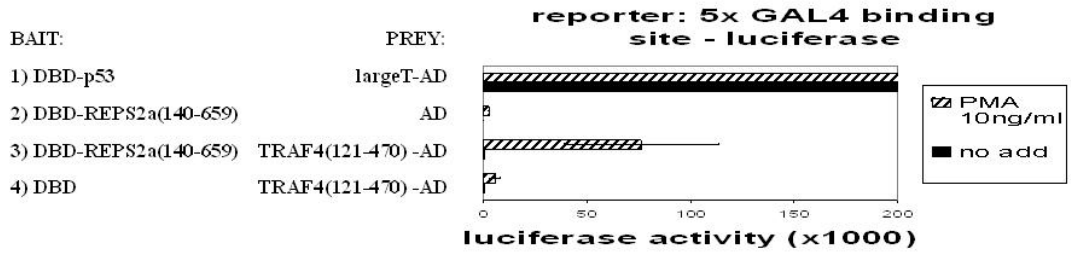


Figure 8 REPS2-TRAF4 interaction in PMA- and non-stimulated LNCaP cells
The assays were conducted as described in Chapter 3 Figure5.

4.5 Signal transducer and activator of transcription 6 (STAT6)

STAT6 belongs to the signal transducers and activators of transcription (STAT) family, which are transcription factors that activate gene transcription in response to a number of cytokines and growth factors. When inactive, STATs are latent cytoplasmic proteins. Janus kinases (JAKs) can activate STATs by tyrosine phosphorylation upon exposure of cells to cytokines or other factors. This tyrosine phosphorylation results in formation of homo- or heterodimers. Dimerized STAT proteins immediately enter the nucleus and bind to specific DNA sequences in the promoter regions of various genes, resulting in gene activation or repression. STAT6 can activate, for example, the promoter of the gene that encodes the cell survival protein BCL2L1 (BCL2-like 1; also known as Bcl-xL) (Masuda et al., 2001).
STAT6 is associated with the NF-κB pathway, since direct interaction between STAT6 and the NF-κB subunit p65 has been demonstrated (Shen & Stavnezer, 1998). Furthermore, it has been reported that STAT6 can inhibit NF-κB activity (Abu-Amer, 2001; Nelson et al., 2003). Moreover, in the context of the present study, it is important to know that STAT6 is active in prostate cancer, analysis of human prostate tissues showed that STAT6 DNA binding activity and STAT6 phosphorylation are increased in tumour tissues compared to normal tissues (Ni et al., 2002), which implies that STAT6 may mediate cell survival in prostate cancer.

Schematic representation of the STAT6

The STAT6 protein consists of 847 amino acid residues. The protein contains a STAT_int domain, which is a protein-protein interaction domain; a STAT_alpha domain, which is a very conserved region among STAT proteins; a STAT_bind domain, which is a DNA binding domain; a SH2 domain, which is a well-known protein-protein interaction domain; and a TAD domain, which is a transactivation domain. Furthermore, a SH3-like domain is indicated, which maybe involved in protein-protein interaction (Figure 9). In this schematic representation of STAT6, an FW motif is depicted that will be discussed below.

The FW motif in STAT6 may bind to the REPS2 EH domain

The yeast two-hybrid assay results presented in Table 1 show that STAT6 (483-847) (Clone 10) binds to full-length REPS2a (1-659) and truncated REPS2a (256-659), but not to the shorter truncated form of REPS2a (451-659), and no binding is found for the NO BAIT control. This indicates that STAT6 binds the EH domain of REPS2. In Chapter 3 it is shown that the EH domain of REPS2 binds NF-κB subunit p65 (Clone 8), probably at the NPF motif in the p65 protein. NPF motifs are considered to be class 1 targets for EH domains (Chapter 1, Paragraph 1.4). The STAT6 (483-847) amino acid sequence was analysed for the presence of such a NPF motif, but this was not found. However, besides the NPF motif, other motifs have been described as binding sequences for an EH domain (Chapter 1, Paragraph 1.4), and one of these is the FW motif. STAT6 (483-847) may have such a FW motif at amino acid positions 514 and 515, where a phenylalanine (F) and a tryptophan (W) are located (see Figure 9). Therefore, we suggest that this FW motif might be the site that binds to the EH domain of REPS2.

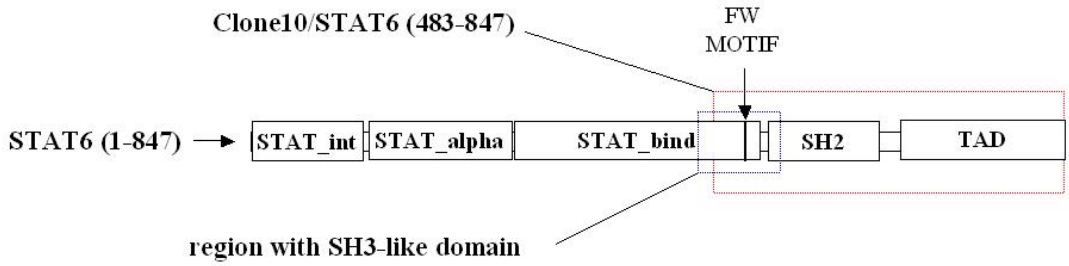
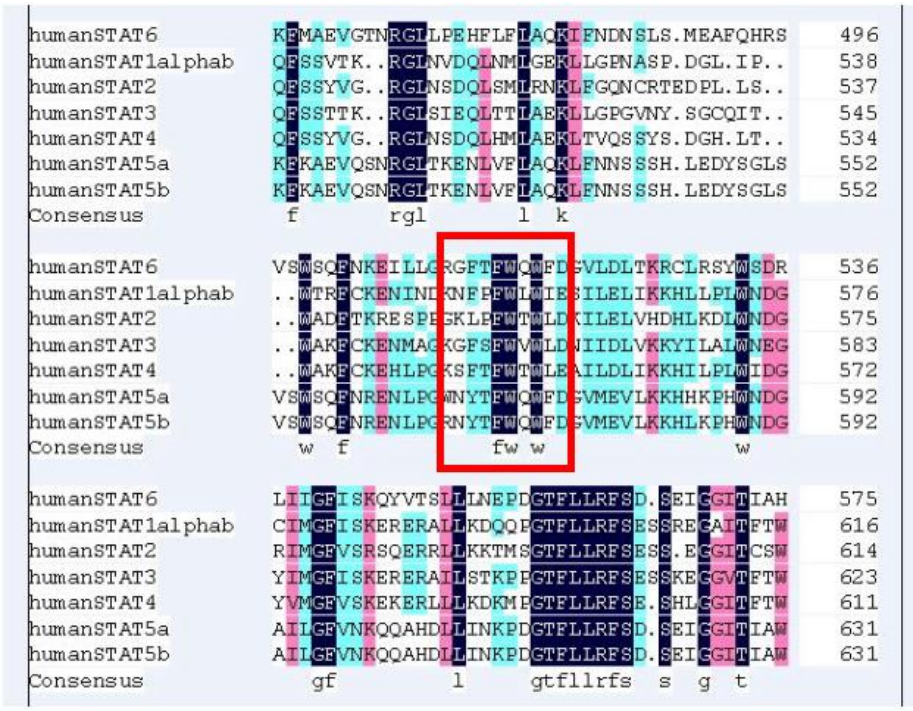


Figure 9 Schematic representation of STAT6
For explanation of the depicted domains and motif, see text in Paragraph 4.5.
The prey amino acid sequence STAT6 (483-847) (See Appendix Table 1 Clone 10) is indicated by a red box.
Note: Most of the domains that are depicted in the schematic representations were determined with the use of the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2003). The position of the TAD in STAT6 was deduced from a schematic representation of STAT6 (Moriggl et al., 1997). The region in STAT6 that contains a SH3-like domain was deduced from schematic representations of STAT6 described in literature (Ihle & Kerr, 1995; Takeda et al., 1997).

There are 7 known STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5a and STAT5b, and STAT6. Alignment of the amino acid sequences of these STATs shows that the candidate FW motif is conserved among the STAT proteins (Figure 10a). This observation indicates that this FW motif may be an important functional site in STAT proteins. On the other hand, structural data showed that the NPF motif of p65 is located in the turn of a loop at the surface of the p65 protein (see Chapter 3, Figure 2b, 2c, 2d) and this is the best position for optimal binding of that motif to an EH domain (de Beer et al., 2000). The structure of a STAT1-DNA Complex (Chen et al., 1998c) showed that the FW motif in STAT1 is not in a similar turn of a loop at the surface of the protein, but located at the end of an α-helix (Figure 10b). Possibly, the FW motif in STAT6 is located in a comparable position. However, this does not exclude that the FW site in STAT6 (or other STATs) may become exposed upon regulatory changes of STAT conformation and activity, and it remains of interest to study further a possible interaction of the FW motif of STAT6 with the EH domain of REPS2. Besides the NPF and FW motifs, other motifs have been identified to be involved in binding to EH domains (Chapter 1, Paragraph 1.4). However none of these other motifs is found in the truncated STAT6 (483-847) amino acid sequence.
Taken together, the results presented in Table1 show that STAT6 binds to the REPS2 EH domain. When indeed the FW motif of STAT6 binds to this domain, it would signify that the REPS2 EH domain is able to bind to the NPF motif in p65 and also to the FW motif in STAT6. This would be similar to the third EH domain of EPS15, for which it has been shown that it can bind to peptides with NPF and FW motifs (Enmon et al., 2000).

A



B

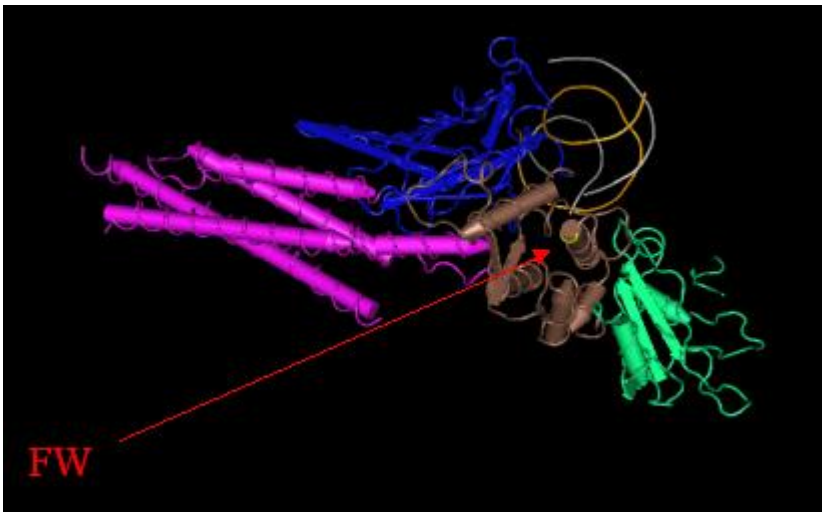


Figure 10 The FW motif is conserved among STATs, but in STAT1 this motif is not located in a turn of a loop

(A) The amino acid sequences from the seven human STATs were aligned using the DNAMAN program. The red box indicates the FW motif.

(B) Structure of STAT1 bound to DNA. The STAT_alpha domain is shown in pink, the STAT_bind domain is shown in blue and brown, and the SH2 domain is shown in green. The FW sequence is highlighted in yellow and indicated with a red arrow. The DNA is shown in orange and grey. The picture was generated using the Cn3d 3D-structure viewer, which is available at the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

4.6 Discussion: REPS2 protein-protein interaction in two-hybrid systems

The yeast two-hybrid screen and assays that are described in the Appendix, Chapter 3 and in this Chapter show binding between REPS2 bait amino acid sequences and several prey amino acid sequences. Excluding false positives, it is to be expected that these interactions can also be demonstrated in other assay systems. For the interaction of REPS2 with p65 and Clone4 (Chapter 3) and TRAF4 (this Chapter), indeed this binding was confirmed using mammalian two-hybrid assays in COS-1 cells. However, in unstimulated LNCaP cells none of the bindings could be demonstrated, whereas the positive control, mammalian two-hybrid binding between p53 and largeT, gave a positive result also for the LNCaP cells. Activation of signalling pathways in LNCaP cells by exposure of the cells to PMA phorbol ester or TNFα resulted in positive signals for the binding of REPS2 to p65, Clone4, and TRAF4 (Chapter 3 and this Chapter). This shows that signalling mechanisms in the mammalian two-hybrid host cells can affect on the observed protein-protein interactions.

Mechanisms in COS-1 and LNCaP cells interfere with REPS2-p65 interactions

Before the bait can bind the prey in yeast and mammalian two-hybrid assay, two important steps take place. First the two hybrids are synthesized in the cytoplasm, and second the two hybrids translocate to the nucleus. At any point in these two steps, different factors may prevent direct interaction between the bait and prey hybrid proteins. Possible mechanisms leading to derailments of binding include: (1) the bait and/or prey hybrid proteins are blocked by protein(s) in the cytoplasm, which prevents translocation to the nucleus; (2) the bait and/or prey hybrid proteins are degraded before they reach the nucleus; (3) the bait and/or prey sequences are modified post-translationally, in cytoplasm and/or nucleus, and this modification inhibits or alters the binding between bait and prey. All three mechanisms can be targets for activated signalling pathways.
The host cells used for the two-hybrid assay (yeast cells, COS-1 cells, or LNCaP cells) is a very important factor in trying to eliminate the mechanisms which interfere with bait-prey interaction. However, exposure of cells - in particular the mammalian cell types - to signalling factors can play a crucial role, exerting pronounced effects on bait-prey interaction.
With regard to differences between yeast and mammalian cells, in the present experiments we observed that the REPS2b bait did bind the p65 (74-551) prey in the yeast two-hybrid assay, but not in the mammalian two-hybrid assay in COS-1 cells (Chapter 3 Figure 3a versus Chapter 3 Figure 4a). Still, REPS2b is appropriate to be used as bait in the mammalian two-hybrid assays in COS-1 cells, since the control, interaction of REPS2b with the Clone4 protein, produced a high binding signal under the same conditions (Chapter 3, Figure 4a). This control assay indicated that the REPS2b bait hybrid is translocated to the nucleus in COS-1 cells, which means that this hybrid protein is not blocked or degraded in the cytoplasm. The related mammalian two-hybrid binding between REPS2 (1-373) and p65 (74-551) produced a high binding signal (Chapter 3, Figure 4a), which shows that the p65 (74-551) prey hybrid also can reach the nucleus. Taken together, this points to the possibility that the REPS2b bait and/or the p65 (74-551) prey are subject to post-translational

modification in COS-1 cells, and such modification would exert an inhibitory effect on the binding between this bait and the prey. The proteins that are responsible for these putative post-translational modifications are not present or active in yeast cells, or the mammalian sequences are not a target for the respective yeast enzymatic machinery. Finally, it cannot be excluded that yeast cells contain endogenous proteins that promote binding between REPS2b and p65 (74-551), and that such proteins are lacking in COS-1 cells.
Six EH domain-containing REPS2 variants were all found to bind to p65 (74-551) in the yeast two-hybrid system (Chapter 3, Table 1). In contrast, in the mammalian two-hybrid system, only three of these EH domain-containing REPS2 variants showed binding to p65 (74-551) in COS-1 cells (Chapter 3, Figure 4a), and none of the REPS2 variants showed such binding in unstimulated LNCaP (Chapter 3, Figure 4b). Also, the REPS2–Clone4 control assays did not show an interaction (Chapter 3, Figure 4b). This suggests that unknown mechanisms in unstimulated LNCaP cells prevent interaction between REPS2 and different prey amino acid sequences.
Lack of expression of the REPS2 bait hybrids and/or the p65 and Clone4 prey hybrids could also be an explanation for absence of binding in unstimulated LNCaP cells, however p53 and large T hybrids seem to be expressed well in LNCaP. Since the constructs encoding the p53 and largeT hybrids use the same vectors as the constructs encoding the REPS2, p65 and Clone4 hybrids it is not likely that lack of expression is the reason for the absence of a binding signal in LNCaP cells (Chapter 3, Figure 4b), however expression of the hybrids has not been monitored on Western blot.

NF-κB pathway regulators might be involved in mechanisms that regulate binding of REPS2 to p65 in LNCaP cells

Posttranslational modification (PTM) of the REPS2 baits and/or the p65 (74-551) prey amino acid sequence, may explain the lack of binding between REPS2 and p65 in unstimulated LNCaP cells (Chapter 3, Figure 4b). Specific proteins that are present in LNCaP cells could carry out PTM of bait or prey or both, and such specific proteins might be regulators of the NF-κB pathway, since p65 is a subunit of the NF-κB complex. Also, activation of the NF-κB pathway might change the activity of specific endogenous proteins in LNCaP cells, resulting in a changed pattern of PTM of bait and/or prey, leading to regain of binding between REPS2 and p65.
This idea was tested by exposure of the LNCaP cells to phorbol ester (PMA), which is an activator of the NF-κB pathway. REPS2-p65 mammalian two-hybrid assays were conducted in the absence or presence of PMA. In the absence of PMA, no REPS2-p65 binding signals could be detected. However, in the presence of PMA, REPS2-p65 binding was detected (Chapter 3, Figure 5a). Indeed, PMA-induced signalling may have changed the activity of regulators of the NF-κB pathway that can affect REPS2-p65 binding at the PTM level. This effect of PMA was specific for REPS2-p65 binding, since the p53-largeT control binding seemed to be unaffected upon stimulation of the LNCaP cells with PMA (Chapter 3, Figure 5a). TNFα, which is another activator of the NF-κB pathway, showed an effect on REPS2-p65 binding that is similar to the effect of PMA (Paragraph 4.2, Figure 4).
Not all the REPS2 baits with an EH domain, when cotransfected with the p65 (74-551) prey, produced comparable binding signal in PMA-stimulated LNCaP cells (Chapter 3, Figure 5a). In contrast, all the REPS2 baits containing the C-terminal part of REPS2, when cotransfected with the Clone4 prey, produced similar binding signals in PMA-stimulated LNCaP cells (Chapter 3, Figure 5b). The pattern of binding

signals in Chapter 3 Figure 5b suggests that the effect of PMA-induced signalling on REPS2-Clone4 binding requires the presence of the C-terminal region of REPS2 and/or the Clone4 prey amino acid sequence. In contrast, the pattern of binding signals in Chapter 3 Figure 5a suggests that PMA-induced signalling in LNCaP cells positively affects REPS2-p65 binding through a site in the N-terminal region of REPS2. The pattern of binding signals furthermore suggests that the C-terminal part of REPS2 has an inhibiting effect on REPS2-p65 binding. A similar effect of the C-terminal part of REPS2 on the binding of REPS2 to p65 was observed in mammalian two-hybrid assays in COS-1 cells (Chapter 3, Figure 4a). This putative regulation of REPS2-p65 binding needs to be studied further.

The REPS2 hybrid and apoptosis

In Chapter 2, we demonstrate that expression of GFP_REPS2 (GFP fused to REPS2) in LNCaP cells leads to apoptosis. Similarly, DBD_REPS2 (GAL4-DBD fused to REPS2, which is the bait hybrid) might induce apoptosis in LNCaP cells during the mammalian two-hybrid assay. Therefore, besides the mechanisms that are described above, apoptosis induced by expression of DBD_REPS2 might explain the lack of a binding signal for REPS2-p65, REPS2-Clone4, and REPS2-TRAF4 interactions in mammalian two-hybrid assays in LNCaP cells (Chapter 3 and this Chapter). However, the fusion proteins DBD_REPS2 and GFP_REPS2 have unique characteristics, which might be important for the apoptosis-inducing ability of REPS2. For example, in contrast to GFP_REPS2, DBD_REPS2 contains a nuclear localisation signal (NLS), which drives the fusion protein into the nucleus, and this may prevent the REPS2 part of the fusion protein from inducing apoptosis. The two fusion proteins are expressed differentially, since the plasmids that encode the fusion proteins contain different promoters. Therefore, the absence of a REPS2-p65 mammalian two-hybrid assay binding signal may not be related to apoptosis induced by the DBD_REPS2. Moreover, REPS2-p65 binding could be detected in TNFα- or PMA-stimulated LNCaP cells, which shows that not all the transfected cells have died from apoptosis. The binding assays in the presence of both signalling molecules resulted in stimulation of REPS2-p65 binding even though TNFα (Sensibar et al., 1995) and PMA (Engedal et al., 2002; Fujii et al., 2000) induce apoptosis in LNCaP cells.

Taken together, the results presented in Chapter 3 and in this Chapter suggest that proteins that are functionally related to the NF-κB pathway may regulate REPS2-p65 binding in LNCaP cells. From literature it is known that NF-κB transcription factors are absent in yeast, and this may explain why none of the REPS2-p65 bindings were found to be suppressed in yeast.

References

References are listed on pages 88-93.

CHAPTER 5

General Discussion and Future Directions

General Discussion and Future Directions

5.1 General Discussion

Introduction

Prostate cancer cells inevitably obtain, after a period of time, the ability to survive androgen ablation therapy (Chapter 1, Paragraph 1.1). Proliferation of these prostate cancer cells has become independent from androgens, and this is referred to as AIPC (androgen-independent prostate cancer). Although several mechanisms have been identified that may contribute to survival of AIPC cells during androgen ablation therapy (see Chapter 1, Paragraphs 1.2 and 1.3), additional mechanisms may exist. Identifying genes that are differentially expressed between ADPC (androgen-dependent prostate cancer; which precedes AIPC) and AIPC can be a good start, to reveal new molecular mechanisms that contribute to the transition from ADPC to AIPC. In a study to find such differentially expressed genes, it was observed that the REPS2 mRNA level in ADPC is much higher than that in AIPC (Chang et al., 1997) (Chapter 1, Paragraph 1.4). The decrease of REPS2 expression in AIPC may play a role in the ability of AIPC to escape from cell death induced by androgen ablation therapy.

How can a decrease in REPS2 expression contribute to survival of AIPC cells during androgen ablation therapy?

A change in REPS2 mRNA level does not necessarily imply that there is a corresponding change in REPS2 protein level. To study the REPS2 protein level in ADPC and AIPC cells, antibodies were raised against REPS2 (Chapter 2). With these antibodies it was determined that the REPS2 protein level correlates well with the relatively high and low REPS2 mRNA level in ADPC and AIPC, respectively (Chapter 2). So, it is warranted to ask the question: how can a decrease in REPS2 protein level contribute to survival of AIPC cells during androgen ablation therapy?
It can be suggested that a decrease in REPS2 protein level in AIPC may put off-balance specific protein-protein interactions in which REPS2 is involved. Some of these protein-protein interactions might control cell death, and might be involved in induction of cell death as it occurs in ADPC cells during conditions of androgen ablation. In AIPC cells in which the REPS2 protein level is markedly decreased, this loss of REPS2 may lead to loss of the cell death response that is normally triggered by androgen ablation. Since REPS2 plays a role in regulating RME (receptor-mediated endocytosis) (Chapter 1, Paragraph 1.4), a decreased REPS2 protein level might result in loss of different REPS2 interactions to proteins that are important for RME. Disturbed RME would lead to decreased downregulation and continued activity of growth factor receptors, which may contribute to survival of AIPC cells during androgen ablation therapy (Chapter 1, Paragraph 1.4).
Besides a role in RME, REPS2 is probably involved also in other cellular processes. Identification of new cellular binding partners for REPS2 can help to find these additional roles for REPS2 in different cellular processes.

Identification of novel REPS2 binding partners

The yeast two-hybrid system was used to identify novel REPS2 binding partners. Indeed, we found that fragments from different human proteins are able to bind physically to amino acid sequences from REPS2 in yeast cells (Appendix and Chapter 3 and 4). Some of the identified interactions were studied further in a more pertinent cellular environment, using mammalian two-hybrid assay, and data obtained with this mammalian cell assay suggest that the protein-protein interactions in which REPS2 is involved are highly regulated (Chapter 4, Paragraph 4.9).

REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interactions

The newly identified REPS2 binding partners: p65, TRAF4, and STAT6, were studied further, since these three proteins are all involved in cell survival and cell death mechanisms. Moreover, these three proteins are all connected to the NF-κB pathway, a signalling pathway which might play an important role in the ability of AIPC to survive androgen ablation therapy (Chapter 4, Paragraph 4.2). In connection with the observations that REPS2 itself plays a role in maintenance of life-death balance in human cells (overexpression of REPS2 was found to induce apoptosis in prostate cancer cells; Chapter 2), it appears that we have identified protein partners that may act together with REPS2 in control of cell survival and cell death.
Apoptosis of AIPC cells that was found to be induced by overexpression of REPS2 might be a consequence of inhibition of the NF-κB pathway, which normally is constitutively active and then promotes cell survival. However, overexpression of REPS2 in ADPC LNCaP cells, which have an inactive NF-κB pathway, resulted in apoptosis (Chapter 2). From this, it can be suggested that REPS2 may affect proteins from additional pathways that are involved in the life-death balance within cells. An obvious candidate protein to be involved in such a pathway is STAT6, as it has been demonstrated that this protein can upregulate the cell survival protein BCL2L1 (Masuda et al., 2001) and is constitutively active in ADPC (Ni et al., 2002).

Signal transduction pathways and REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interactions

In LNCaP cells, REPS2-p65 and REPS2-TRAF4 interactions seem to be induced by signalling pathways that are sensitive to the NF-κB pathway activator PMA (Chapter 3 and 4). The effect of the phorbol ester PMA on REPS2-STAT6 binding has not been investigated yet. In addition, signalling pathways that are sensitive to TNFα, which is another NF-κB pathway activator, seem to stimulate REPS2-p65 interaction in LNCaP cells (Chapter 4). The effect of TNFα sensitive signalling pathways on REPS2-TRAF4 and REPS2-STAT6 binding in LNCaP cells remains to be investigated. Nevertheless, this experimental data supports the connection of REPS2 to the NF-κB pathway.
PMA and TNFα activate endogenous signal transduction pathways; however, the pathways that lead to activation of the NF-κB pathway are not fully understood. By using wortmannin (Ui et al., 1995), that acts as a selective inhibitor of phosphoinositide 3-kinase (PI3K), it has been demonstrated that PI3K plays a role in NF-κB activation by PMA, but not by TNFα in U937 cells (human histiocytic

lymphoma) (Manna & Aggarwal, 2000). This indicates that NF-κB is activated by different pathways when exposed to different activators.
The EGF receptor and the downstream signal transduction pathways as they are depicted in Chapter 1 (Figure 2) are implicated in regulation of NF-κB dependent transcription. EGF induces a pathway that leads to activation of NF-κB, and this event is blocked by Ly294-002, an inhibitor of PI3K, suggesting a role of PI3K in activation of NF-κB by EGF (Biswas et al., 2000). The small GTPase RAS, which has a function downstream of the EGF receptor (see Chapter 1, Paragraph 1.4), also is connected to the NF-κB pathway. It has been shown that NF-κB activation is required to suppress apoptosis induced by oncogenic RAS, which is a constitutively active form of RAS (Mayo et al., 1997). Furthermore it has been reported that oncogenic RAS enhances NF-κB transcriptional activity through RAF-dependent and RAF-independent mitogen-activated protein kinase signalling pathways (Norris & Baldwin, 1999). Recently, it has been demonstrated that constitutive NF-κB DNA-binding activity in AML (acute myelogenous leukaemia) is frequently mediated by a RAS/PI3K/AKT-dependent pathway (Birkenkamp et al., 2004). Expression of activated RAL in quiescent rodent fibroblasts is sufficient to induce activation of NF-κB-dependent gene expression (Henry et al., 2000). The human CDC42 and RAC1 proteins efficiently induce the transcriptional activity of NF-κB by a mechanism that involves phosphorylation of IκB and translocation of p50/p50 and p50/p65 dimers to the nucleus, but independent of RAS and RAF (Perona et al., 1997). In addition, RAC1 regulates interleukin 1 (IL1)-induced NF-κB activation in an IκB-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression (Jefferies & O'Neill, 2000). Figure 1 shows the signal transduction pathways schematically. It should be noted that the pathways are drawn for a single cell, although they were identified in different cell types and species. This needs to be taken into account, also for interpretation of literature data.
The REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interactions might be involved in EGF-, RAS-, RAF-, PI3K-, RAL-, CDC42-, and RAC1-mediated NF-κB pathway regulation, since REPS2 is connected to all these proteins (Chapter 1, Figure 2).

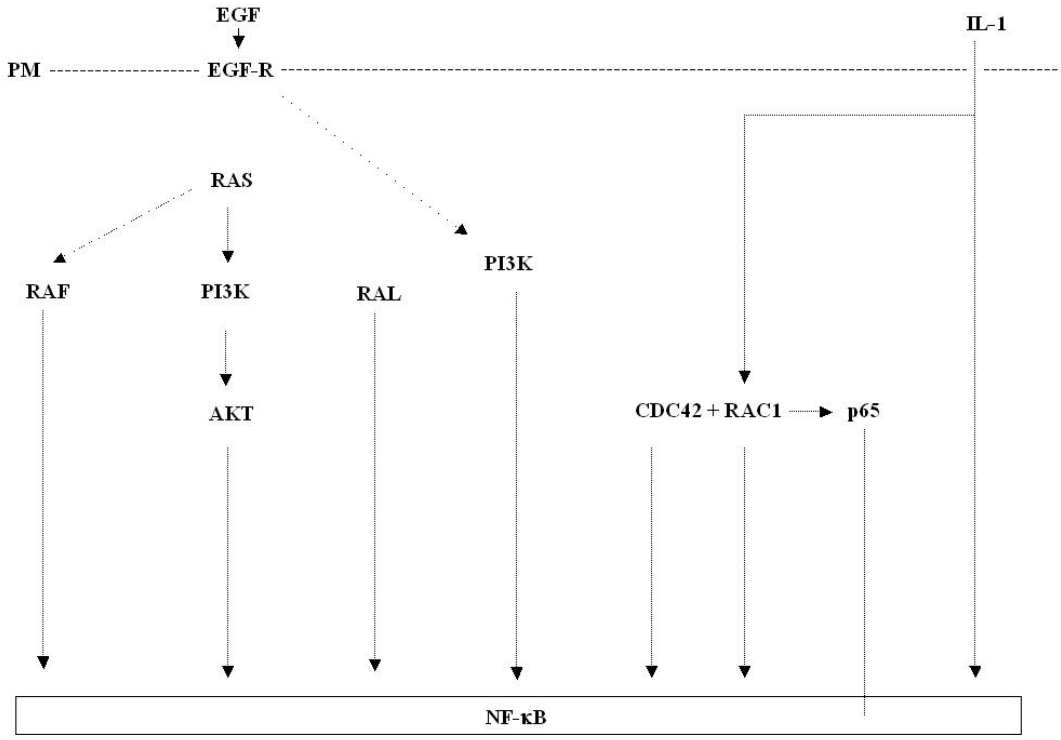


Figure 1 Schematic representation of REPS2 related pathways that are involved in regulation of NF-κB dependent transcription
For explanation see text in Paragraph 5.1.
PM: plasma membrane.

Decreased REPS2 protein level may shift the life-death balance of AIPC cells towards life, possibly through a stimulatory effect on NF-κB activity

Several reports in literature describe that NF-κB pathway activity is highly increased in AIPC (Chen & Sawyers, 2002; Gasparian et al., 2002; Palayoor et al., 1999). Little is known about factors which might control NF-κB pathway activity in ADPC and AIPC (Chapter 4, Paragraph 4.2). The present findings on protein-protein interactions in which REPS2 takes part, provide additional information that is relevant to understand control of NF-κB activity in prostate cancer. The REPS2 protein level is much higher in ADPC compared to AIPC (Chapter 2). In addition, it is now known that REPS2 is able to physically interact with proteins that are connected to the NF-κB pathway, namely p65, TRAF4, and STAT6 (and possibly other proteins, see Paragraph 5.2). Therefore, we suggest that the decrease in REPS2 protein level may contribute to a highly active NF-κB pathway in AIPC.
The cytoplasmic fraction of an androgen-dependent LAPC-4 xenograft tumour has been found to contain relatively high p50 and p65 protein levels, whereas the cytoplasmic fraction of its androgen-independent counterpart contained only very low p50 and p65 protein levels; the nuclear extracts of these tumours showed the inverse (Chen & Sawyers, 2002). This suggests that the p50/p65 dimer in ADPC is prevented from translocation to the nucleus. REPS2 is mainly located in the cytoplasm (Chapter

2). Possibly, a high level of REPS2 in ADPC keeps the NF-κB p65 subunit into the cytoplasm, preventing it from translocating to the nucleus, and therefore preventing it from binding to DNA and activating gene transcription.
The REPS2-TRAF4 interaction might play a role in preventing translocation of p65 to the nucleus, since inhibiting activity of TRAF4 towards the NF-κB pathway has been demonstrated. Activity of the common neurotrophin receptor, which belongs to the TNF receptor superfamily, is connected to activation of the NF-κB pathway (Ye et al., 1999). In HEK 293T cells, TRAF4 exerts an inhibiting action on neurotrophin receptor-induced activation of NF-κB (Ye et al., 1999). Interestingly, in primary T-cells and Jurkat cells stimulated with TNFα or PMA, TRAF4 mRNA was rapidly upregulated (Glauner et al., 2002). Together, the results from these studies suggest that TRAF4 mediates a negative feedback loop mechanism that represses NF-κB activity.
The REPS2-STAT6 interaction might play a role in preventing p65 from translocation to the nucleus, since STAT6 is also involved in NF-κB regulation. Expression of STAT6 was shown to alter p65 nuclear occupancy by affecting the import rate and hence the overall maximal level of p65 translocation (Nelson et al., 2003). Activating STAT6 with IL4 (interleukin 4), prior to activation of NF-κB, significantly increased this inhibition. It was demonstrated that STAT6 inhibited TNFα-mediated NFKBIA (which is a IκB inhibitor of NF-κB) phosphorylation and degradation (Nelson et al., 2003).
The NF-κB pathway is known to play a crucial role in control of cell survival (Beg & Baltimore, 1996; Hoberg et al., 2004; Karin, 1998; Levkau et al., 1999; Van Antwerp et al., 1996; Wang et al., 1996; Yeung et al., 2004). Hence, our findings about interaction of REPS2 with p65, TRAF4, and STAT6 emphasize a possible role of REPS2 in control of prostate cell apoptosis. If, and through what kind of mechanisms exactly, loss of REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interactions might lead to a highly increased NF-κB pathway, needs to be studied. But if downregulation of REPS2 indeed contributes to upregulation of NF-κB activity, then downregulation of REPS2 will contribute to an additional cell survival mechanism in AIPC. The decrease in REPS2 protein level might also affect other proteins interactions that regulate cell survival mechanisms, and such an additional positive control of cell survival may contribute to the ability of AIPC to survive androgen ablation therapy (see also Chapter 3, Figure 7).
Different mechanisms, AR dependent and AR independent, have been identified so far that may contribute to the ability of AIPC to survive androgen ablation therapy-induced cell death (Chapter 1, Paragraphs 1.2 and 1.3). These mechanisms may act independent or in combination. Despite all these identified mechanisms, there is no doubt that other mechanisms exist. Nevertheless, successful interference in one or more mechanism(s) may already be sufficient to push the life-death balance within AIPC cells towards death.

5.2 Future directions

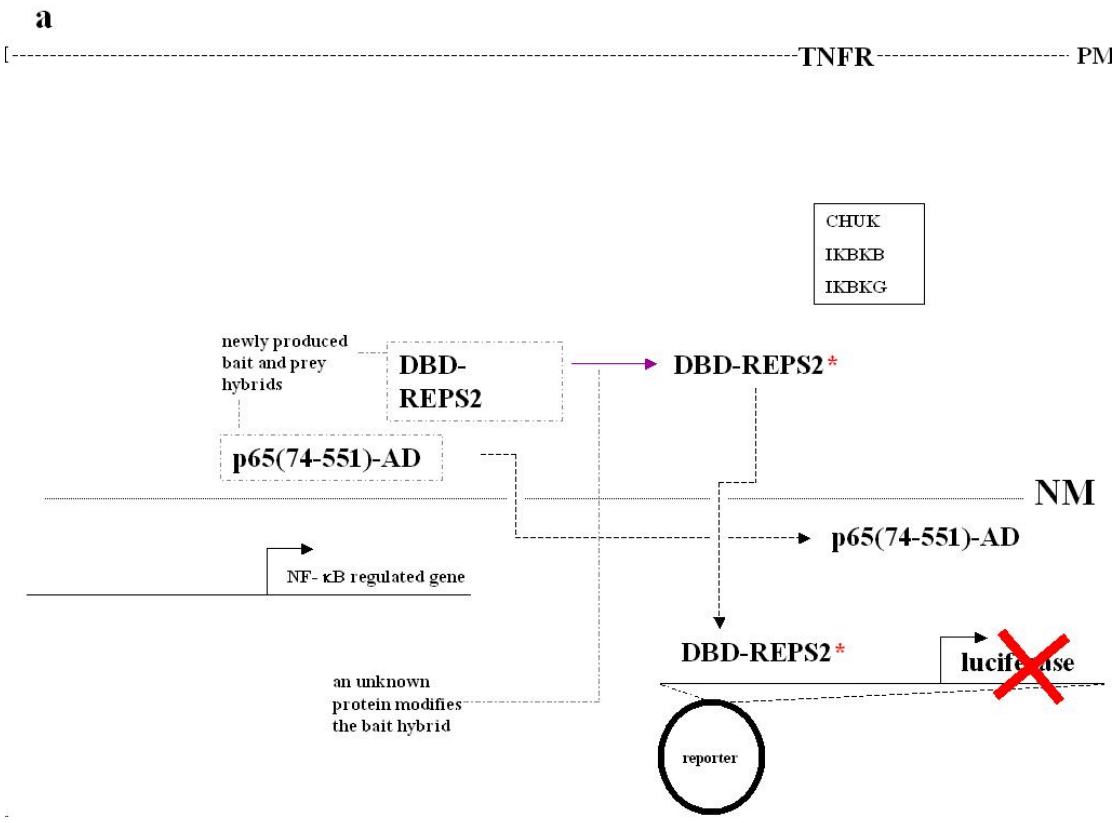
Identification of other REPS2 protein partners may increase our knowledge of the putative role of REPS2 in control of the life-death balance of cells

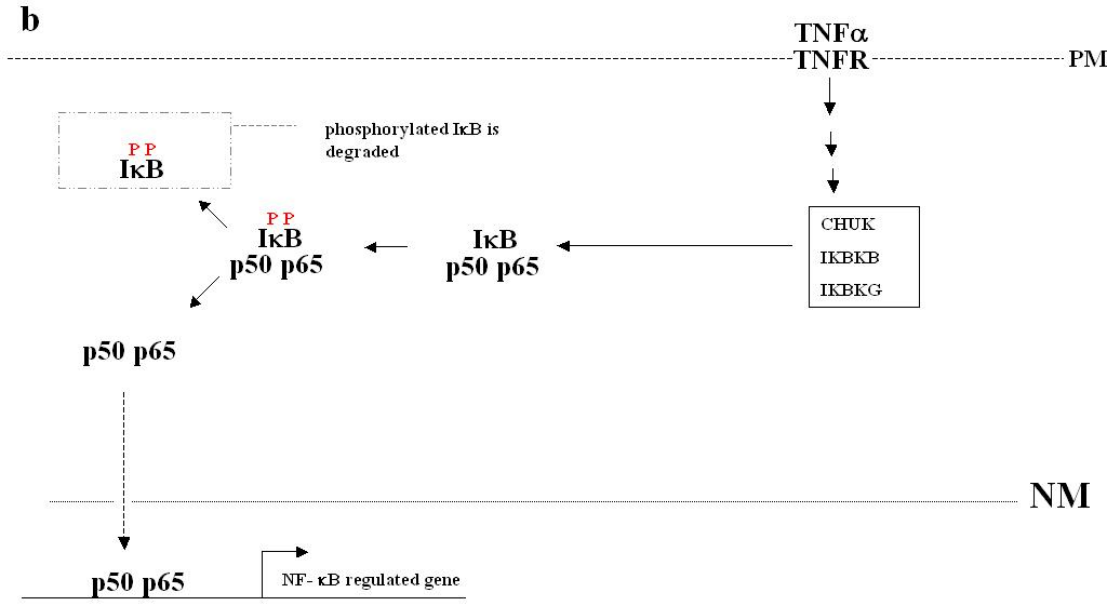
Besides identification of p65, TRAF4, and STAT6, amino acid sequences from other proteins were identified to bind physically to REPS2 in the two-hybrid screen (see Appendix, Table 1). Further research is required to obtain evidence for a possible role of these other proteins in a regulatory network which includes REPS2. Similarly, it remains important to identify the preys from several uncharacterized positive yeast colonies (see Appendix) or from newly obtained positive colonies. Considering that three proteins could more or less directly be connected to the NF-κB pathway, out of only sixteen proteins sequences that were identified in the yeast two-hybrid screen as REPS2 binding partner (Appendix), it is not unlikely that other NF-κB related proteins will be identified as REPS2 protein partner.

Identification of NF-κB regulators that may control REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interactions

REPS2-p65 mammalian two-hybrid interaction is highly regulated in LNCaP cells (Chapter 4, Paragraph 4.9). It is not clear how this regulation takes place, but it might be that binding of REPS2 to p65 in LNCaP cells is prevented through post-translational modification of REPS2 immediately upon translation of REPS2. Controlled action of specific endogenous proteins might abolish post translational modifications and thereby reverse the REPS2-p65 binding inhibition.
Figure 2a shows, schematically, a mammalian two-hybrid assay in LNCaP cells with REPS2 as bait and p65 as prey. Hypothetically, an unknown protein may modify the REPS2 bait directly after it has been synthesized. The REPS2 bait then may lose its ability to bind to the p65 prey. This might explain the failure to show a REPS2-p65 mammalian two-hybrid binding signal in LNCaP cells (Chapters 3 and 4).
We have suggested that activation of the NF-κB pathway could stimulate the activity of endogenous proteins that have the ability to counteract the REPS2-p65 binding inhibition (Chapters 3 and 4). Indeed, it could be demonstrated that TNFα sensitive signalling pathways stimulate REPS2-p65 binding in LNCaP cells (Chapter 4, Paragraph 4.2). Several steps of TNFα signalling that lead to NF-κB activation are known (Hayden & Ghosh, 2004). Binding of TNFα to its receptor results in activation of signal transduction cascades, which leads to, among others, activation of the IKK complex, which consists of CHUCK, IKBKB, and IKBKG. The activated IKK complex subsequently phosphorylates the IκB protein, which is bound to the NF-κB dimer. This phosphorylation leads to dissociation of IκB from the NF-κB dimer, which results in translocation of the NF-κB dimer to the nucleus. Figure 2b shows schematically how TNFα activates the NF-κB pathway.
Figure 2c represents a mammalian two-hybrid assay similar to that in Figure 2a, but the host LNCaP cells were stimulated with TNFα. Hypothetically, it is shown that TNFα sensitive pathways modify the modified REPS2 bait back to wild type REPS2, which enables the bait to bind the p65 (74-551) prey.

For NF-κB, several well-characterized inhibitors have been identified, which can be used to get insight into the TNFα signalling sensitive pathways that play a role in stimulation of REPS2-p65 binding. The anti-inflammatory drug aspirin (acetylsalicylic acid), for example, could inhibit TNFα-induced NF-κB pathway activation (Kopp & Ghosh, 1994), through specific inhibition of IKBKB kinase activity (Yin et al., 1998). Aspirin may be used to dissect TNFα sensitive pathways that are important for stimulation of mammalian two-hybrid REPS2-p65 binding in LNCaP cells. The mammalian two-hybrid assay that is shown in Figure 2c can be conducted in the presence or absence of aspirin, to see if TNFα-stimulated signal transduction pathways upstream of IKBKB (green) or downstream of IKBKB (blue) are important for REPS2-p65 binding. Figure 2d shows a possible outcome of the assay in the presence of aspirin, from which can be concluded that TNFα-stimulated signal transduction pathways upstream of IKBKB are important for REPS2-p65 interaction in LNCaP cells.
Similar experiments can be conducted with other signal transduction pathway activators and inhibitors (proteins and compounds), which may provide insight in the pathways that are important for REPS2-p65, REPS2-TRAF4, and REPS2-STAT6 interaction in LNCaP cells.




b
TNFα
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CHUK
IKBKB
IKBKG
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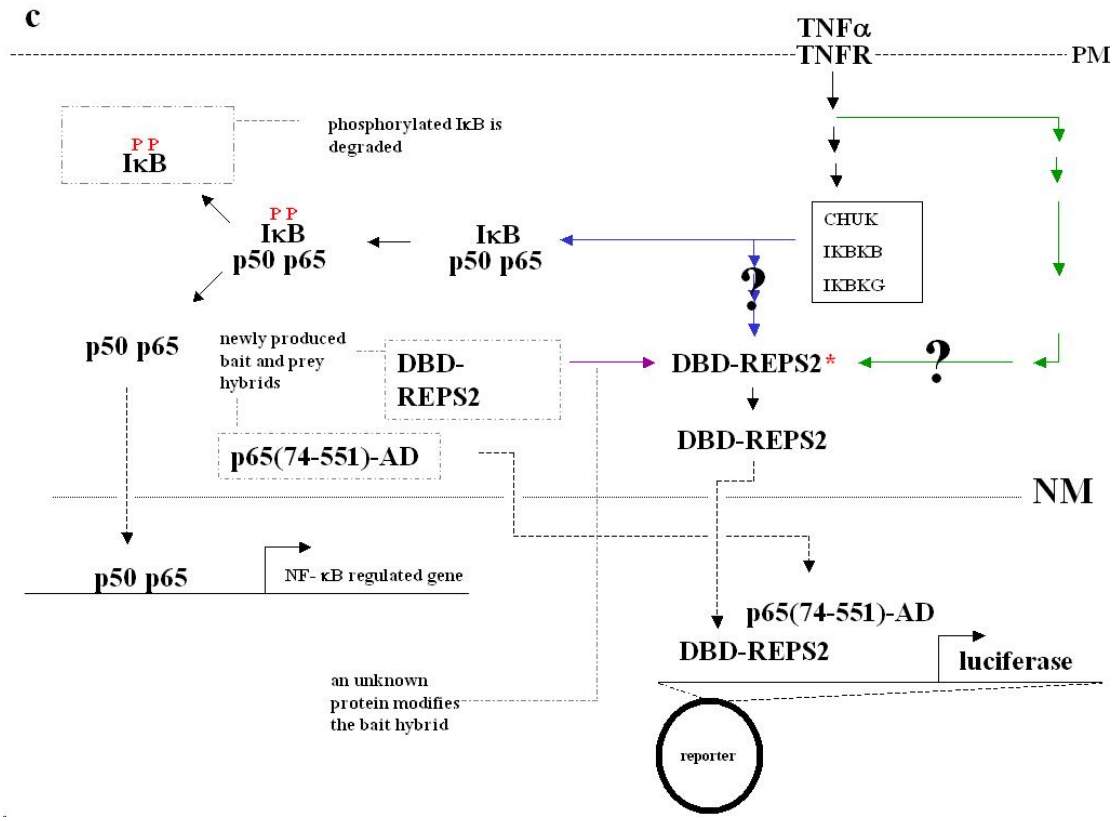
NM
p50 p65
NF- κB regulated gene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CHUK
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p50 p65
newly produced bait and prey hybrids
DBD-REPS2
DBD-REPS2*
DBD-REPS2
p65(74-551)-AD
NM
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NF- κB regulated gene
p65(74-551)-AD
DBD-REPS2
luciferase
an unknown protein modifies the bait hybrid
reporter

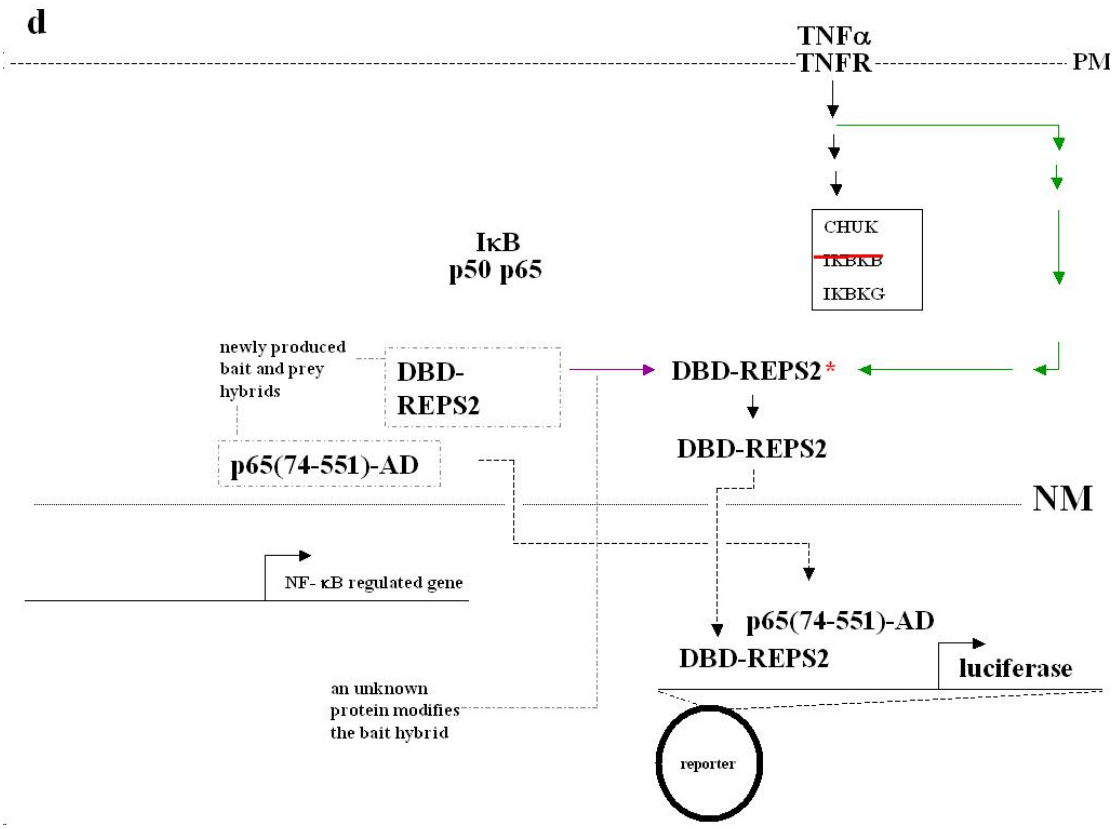

Figure 2 Dissection of TNFα sensitive pathways that are important for REPS2-p65 interaction

(a) Schematic representation of a mammalian two-hybrid assay in unstimulated LNCaP cells. Hypothetically, it is shown that an unknown protein modifies the REPS2-bait amino acid sequence directly after it has been synthesized (the modified REPS2-bait is indicated with a red asterisk). As a result of this modification the REPS2 bait amino acid sequence is unable to bind to the p65 (74-551) prey amino acid sequence. For more explanation, see text in Paragraph 5.2.

(b) Several steps of a TNFα-induced NF-κB pathway are shown.

(c) Schematic representation of a mammalian two-hybrid assay in TNFα stimulated LNCaP cells. The mammalian two-hybrid assay is similar to the assay that is shown in Figure 2b, although in the assay represented here the cell is stimulated with TNFα.

Green and blue arrows indicate TNFα sensitive pathways upstream and downstream, respectively, of IKBKB.

(d) An assay that is similar to the assay shown in Figure 2c, but conducted in the presence of aspirin (inhibition of IKBKB).

For more explanation see text in Paragraph 5.2. For explanation on two-hybrid systems see the Appendix.

PM: plasma membrane; NM: nuclear membrane.

REPS2-mediated inhibition of the NF-κB pathway: is REPS2 a target for therapeutic intervention?

REPS2 may have strong inhibitory activity towards the NF-κB pathway, but such inhibitory activity is probably very sensitive and determined by other factors (Chapter 4, Paragraph 4.2). Inhibitory activity might be dependent on post-translational modifications of REPS2, carried out by different upstream pathways (Figure 3). The activity of these upstream pathways would determine the inhibitory activity of REPS2 towards the NF-κB pathway (Figure 3). It will be of much interest to find out which pathways upstream of REPS2 are important to control the inhibitory activity of REPS2 towards the NF-κB pathway. Analysis of the pathways that play a role in binding of REPS2 to p65, TRAF4, and STAT6, as described in the previous part of this Paragraph, may provide specific information on signal transduction pathways upstream of REPS2. This information can help to find membrane-permeable compounds (MPCs) that can be used to manipulate signal transduction pathways upstream of REPS2 that are important for its inhibiting activity towards the NF-κB pathway.
Cancer cells exposed to a specific combination of MPCs may transform endogenous REPS2, or externally introduced REPS2, into a very potent and specific inhibitor of the NF-κB pathway. MPCs include common drugs such as aspirin and ibuprofen, or components in food products, such as Genistein and grape seed extract. Therefore, knowledge of REPS2- / MPCs-mediated inhibition of the NF-κB pathway might be of direct importance for cancer therapy, as discussed herein for prostate cancer. A pronounced, or perhaps even a moderate decrease of NF-κB activity in AIPC, obtained through REPS2-/MPCs-mediated therapeutic intervention, in combination with androgen ablation therapy or another anti-cancer therapy, might evoke a shift in the life-death balance in AIPC towards death of the cells.

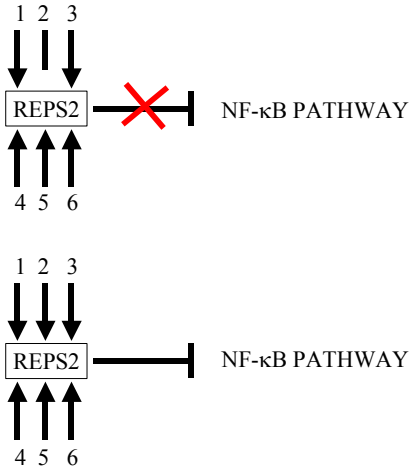

Figure 3 REPS2 and inhibition of the NF-κB pathway
The figure hypothetically shows that the inhibiting activity of REPS2, towards the NF-κB pathway, is controlled by six endogenous signal transduction pathways (1-6). By targeting either one of these upstream pathways this inhibiting activity of REPS2 might me manipulated.

References

References are listed on pages 88-93.

APPENDIX

Yeast two-hybrid screening of an expression library with REPS2 as bait, and a brief description of the mammalian two-hybrid system

Appendix: Yeast two-hybrid screening of an expression library with REPS2 as bait, and a brief description of the mammalian two-hybrid system

Introduction

The REPS2 protein may have several cellular protein partners, since its amino acid sequence has some well-known regions that are possibly involved in protein-protein interaction. One important region is the EPS15 homology (EH) domain (Chapter 3). The EH domain forms a hydrophobic pocket at the surface of the REPS2 protein, wherein amino acid sequences of target proteins can bind. Furthermore, a coiled-coil protein-protein interaction domain and four proline-rich motifs were identified in REPS2 (see also Chapter 1, Paragraph 1.4).
In this Appendix, we discuss several aspects of the yeast two-hybrid system, which was used to identify and study protein-protein interactions. The full length REPS2 protein (REPS2a) was used as a bait, to catch preys from a human prostate cDNA expression library. This resulted in identification of several candidate partners, including NF-κ B subunit p65 (Chapters 3 and 4) and TRAF4 and STAT6 (Chapter 4). These proteins may represent physiological binding partners of REPS2a. Several of the identified interactions were analysed further using the yeast two-hybrid system, and a few of these were confirmed in the mammalian two-hybrid system, which is briefly described at the end of this Appendix.

REPS2 as bait in the yeast two-hybrid system

The yeast two-hybrid system is a technique to identify and study protein-protein interactions, including mammalian proteins, in yeast cells (Fields & Song, 1989). The system can be applied in two ways: two-hybrid screen and two-hybrid assay. In a yeast two-hybrid screen, a known protein, or protein domain, of interest is used as bait to catch preys, which are unknown protein sequences that are able to bind physically to the bait amino acid sequence in yeast cells. The bait sequence is fused to the DNA-binding domain (DBD) of GAL4, which is a yeast transcription factor. This fusion protein is one of the two hybrids in the system. The other hybrid comes from the expression library that is screened. In that library, unknown mammalian protein sequences are fused to the activation domain (AD) amino acid sequence of yeast GAL4. Yeast cells that contain the bait hybrid (Figure 1a) are transformed with the cDNA library (Figure 1b). When indeed a clone from the expression library encodes a protein that interacts with the bait, this will bring the DNA-binding and activation domains of GAL4 within one complex, resulting in reconstitution of GAL4 transcription factor activity, which then activates marker genes in the yeast genome (Figure 1b). These marker genes can be used to select yeast cells in which bait-prey interaction occurs. Prey vectors are subsequently isolated from selection positive yeast colonies, and the cDNA sequences can be determined.
In a yeast two-hybrid assay the yeast two-hybrid system is applied to study protein-protein interaction. A known protein, or protein domain, of interest is used as bait and a known protein, or protein domain of interest is used as prey.

In Chapter 2, it is described that REPS2 occurs as a longer form, which was named REPS2a, and a shorter form, which was named REPS2b. We have used REPS2a as bait in the yeast two-hybrid screen.

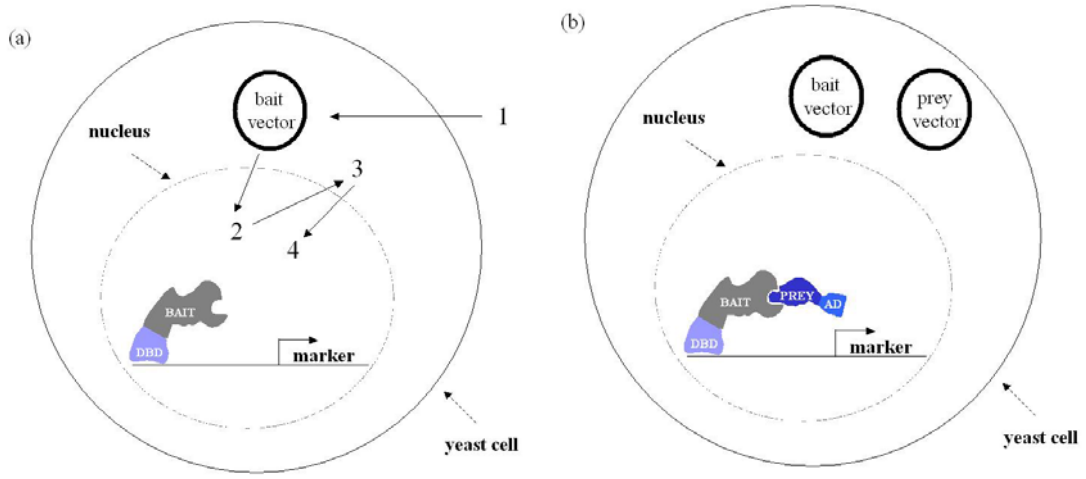

Figure 1 Schematic representation of the yeast two-hybrid system
(a) The picture represents a yeast cell that has been transformed with a plasmid that encodes the bait hybrid. Step 1 shows the intake of the plasmid (bait vector) during the transformation of the yeast cell. This bait vector is transcribed to produce mRNA (2) and the mRNA is translated to produce the bait-hybrid (3). The bait-hybrid is driven to the nucleus by the nuclear localisation signal (NLS) that is present in the amino acid sequence of the GAL4-DBD (4). The bait-hybrid binds specifically through its GAL4-DBD to a binding site in the promoter of a marker gene that is present in the genome of the yeast cell. The AH109 yeast strain, which is the strain we used in the screen, has a *lac*Z-, a histidine-, and an adenine-marker in its genome.
Yeast cells that contain this bait-construct can be made competent (ready to take up plasmids) and subsequently be used to screen a prey cDNA construct library (see Figure 1b)
(b) The yeast cells depicted in Figure 1a were made competent and used to screen a prey-construct library. The picture shows that one prey-construct has entered the yeast cell. The steps that are described for the bait-hybrid (see Figure 1a) occur also for the prey hybrid. In the Figure, it is shown that the amino acid sequence from the prey binds to the amino acid sequence from the bait. This reconstitutes the GAL4 transcription factor, what subsequently results in activation of the marker gene.

REPS2a is appropriate as bait in the yeast two-hybrid system

The BD Matchmaker Two-Hybrid System 3 (BD Biosciences Clontech) was used for the yeast two-hybrid screen and the additional yeast two-hybrid assays. Compared to yeast strains from other yeast two-hybrid systems, the genome of the yeast strain in the Matchmaker System encodes an extra adenine growth selection marker. This adenine marker is not leaky, in contrast to other markers such as the histidine- and the β-galactosidase-markers (*lac*Z), meaning that activation of the adenine marker is very stringent and should occur only after the prey binds to the bait.

Two important conditions for successful application of the yeast two-hybrid system are: (1) the bait hybrid should not have intrinsic transcriptional activity, and (2) the bait hybrid should be properly expressed in the yeast strain. To test the first condition, yeast cells containing the REPS2a bait (AH109-REPS2a) were plated on medium that lacks tryptophan, and on medium that lacks tryptophan AND adenine. It was found that the AH109-REPS2a yeast strain was able to grow on medium that lacks tryptophan, which means that the yeast strain indeed contained the plasmid that encodes the bait hybrid, since this plasmid contains a sequence encoding the tryptophan selection marker. However, the AH109-REPS2a yeast strain was unable to survive on medium that lacks tryptophan AND adenine, which means that the REPS2a bait hybrid (Figure 2a) does not have intrinsic transcriptional trans-activating activity. To test the second condition, cell extract from the AH109-REPS2a strain was analysed on Western blot. It was found that the REPS2a bait-hybrid was properly expressed in the yeast strain (Figure 2b).

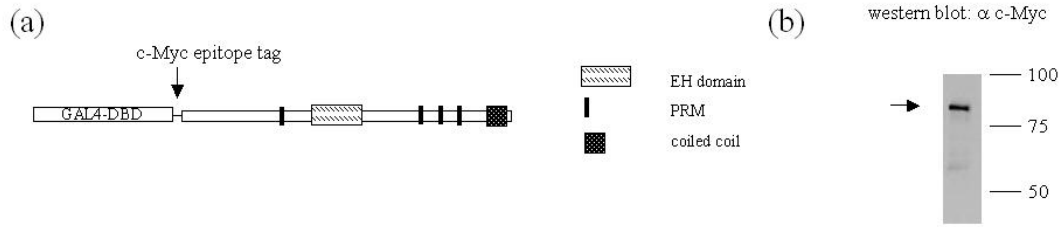

Figure 2 REPS2a bait hybrid is expressed well in the AH109-REPS2a yeast strain
(a) Schematic representation of the REPS2a-bait. (b) Cell extract from the AH109-REPS2a strain on Western blot, where c-Myc epitope antibody detects a protein band of about 92 kD. The size of the protein band corresponds to the calculated molecular mass of the REPS2 bait hybrid (REPS2 is a protein of 71 kD and the GAL4-DBD has a molecular mass of 21 kD).

Different protein sequences were found to bind to the REPS2a bait in the yeast two-hybrid screen

The AH109-REPS2a yeast strain was made competent and transformed with a human prostate cDNA expression library (Figure 3). To determine the number of transformants, a small part of the transformation mix was plated on medium that lacks tryptophan AND leucine. AH109-REPS2a cells that are transformed with a prey-encoding plasmid from the library gain the ability to produce leucine, since there is a leucine marker gene in the prey plasmid, which gives them the ability to survive on plates that lack leucine. In this way the total number of transformants was determined to be approximately 52,000. After 3 days, 20 colonies were observed on the selection plates, that lack not only tryptophan AND leucine but also histidine AND adenine (Table 1, Clones 1–20). In the yeast cells from these colonies, the adenine marker was activated, which indicates bait-prey interaction. Besides the adenine marker, the AH109-REPS2a yeast strain contains a gene encoding a histidine growth selection marker, but this marker was found to be leaky (data not shown), and therefore further ignored. Ten days after the transformation, a total number of 168 colonies were observed on the selection plates, and from 25 of these colonies the prey plasmid was isolated. The prey insert sizes were estimated by restriction enzyme analysis (Table

1). Subsequently, the DNA sequences of the prey inserts were determined and the proteins that they encode were identified (Table1).

Table 1
Preys that were identified to bind to the REPS2a bait in the yeast two-hybrid screen

Clone:	Estimated insert size:	The prey represents:	Gene description:	Part of the full length protein that is fused to GAL4-AD (1)	Sequence Accession ID:
1	500	AMY1A	amylase, alpha 1A; salivary	370 – 551	NM_004038
2	1100	DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	366 - 614	NM_004396
3	800	-	(GAL4-AD in frame with an ORF from the SFRS5 cDNA)	-	NM_006925
4	1800	CDK5RAP2	CDK5 regulatory subunit associated protein 2	1432 - 1893	NM_018249
5	500	AMY1A	amylase, alpha 1A; salivary	364 – 551	NM_004038
6	1400	COL1A1	collagen, type I, alpha 1	1086-1464	NM_000088
7	800	LAMR1	laminin receptor 1 (ribosomal protein SA, 67kDa)	56 - 295	NM_002295
8	1800	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of κ light polypeptide gene enhancer in B-cells 3, p65 (avian)	74-551	NM_021975
9	1800	FN1	fibronectin 1	2008 - 2477 (2)	NM_212482
10	1800	STAT6	signal transducer and activator of transcription 6	483 - 847	NM_003153
11	1100	CTSL	cathepsin L	90 - 333	NM_145918
12	1100	CPE	carboxypeptidase E	295 - 476	NM_001873
13	1800	FN1	fibronectin 1	2015 – 2477	NM_212482
14	1200		ND (problem with sequencing the insert)		
15	1200		ND (problem with sequencing the insert)		
16	1200	DDX17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	355 - 650	U59321
20.3	1100		ND (problem with sequencing the insert)		
22	1900	COL1A1	collagen, type I, alpha 1	793-1464	NM_000088
23	1600	CPE	carboxypeptidase E	203 - 476	NM_001873
25	600	-	(GAL4-AD in frame with an ORF from the AZGP1 cDNA)	-	NM_001185
28	700	GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	106 - 359	AF493900
38	700	LAMR1	laminin receptor 1 (ribosomal protein SA, 67kDa)	124 - 295	NM_002295
41	1600	NOTCH2	Notch homolog 2 (Drosophila)	38 – 2471 (3)	NM_024408
43	ND	RBM4	RNA binding motif protein 4	29 - 366	NM_002896
44	1500	TRAF4	TNF receptor-associated factor 4	121 - 470	X80200

The screening was performed as described in the BD Matchmaker Two-Hybrid System 3 manual, with the exception that carrier DNA from salmon testis was used (Sigma-Aldrich Corporation, St Louis, MI, USA). Prey plasmids were isolated according to the protocol described by Hoffman & Winston (1987).
Notes:
(1) In most cases only the first part of the prey insert has been sequenced. Reverse transcription was oligo-dT primed when the cDNA expression library was constructed (Figure 3), and therefore it is assumed that the prey encodes the complete C-terminal part of the full length protein.
(2) This prey hybrid has a sequence of 5 amino acid residues, between the GAL4-AD and the FN1 (2008 - 2477) sequence, that does not correspond to FN1 (2003-2007).
(3) The expected protein fragment size does not correspond with the estimated prey insert size.
ND = Not Determined
Clones that contain fragments from the same gene product are indicated by the same colour.

Figure 3 Matchmaker cDNA expression library
Reverse transcription of human prostate mRNA was oligo-dT primed, which means that the mRNA was transcribed from the 3' end. Because of this, the cDNA will encode the C-terminal part of the protein or the whole protein. (mRNA source: normal, whole prostate glands pooled from 20 Caucasians, ages 20-58; cause of death: trauma).

The mammalian two-hybrid system

For mammalian proteins, studies on protein-protein interaction in the yeast two-hybrid system (screen and assay) may yield non-physiological results. The yeast cellular environment may not allow mammalian proteins to reach a conformation, similar to the conformation in a mammalian cell. Therefore, using the mammalian two-hybrid system in a protein-protein interaction assay can provide additional information. Whereas the yeast two-hybrid system can be used for screening a library and for individual protein-protein interaction assays, the mammalian two-hybrid system can only be used for the individual protein-protein interaction assays.
Figure 4 shows the mammalian two-hybrid system schematically. Step by step, it involves (1) a suitable cell line is cotransfected with the plasmids that encode the bait and prey hybrids, along with the GAL4-sensitive luciferase reporter plasmid; (2) the bait and prey hybrids contain a nuclear localisation signal (NLS), so that the hybrids will translocate to the nucleus; (3) the bait hybrid will bind via its GAL4-DBD to a specific site that is located upstream the luciferase marker gene in the reporter plasmid; (4) binding of the prey amino acid sequence to the bait amino acid sequence brings the VP16-AD of the prey hybrid into proximity of the GAL4-DBD, and this results in transcription of the reporter gene; (5) transcription of the luciferase reporter gene will result in production of the enzyme luciferase, which can be optically measured using a luciferase substrate. We have used the mammalian two-hybrid system, as described in Chapters 3 and 4.

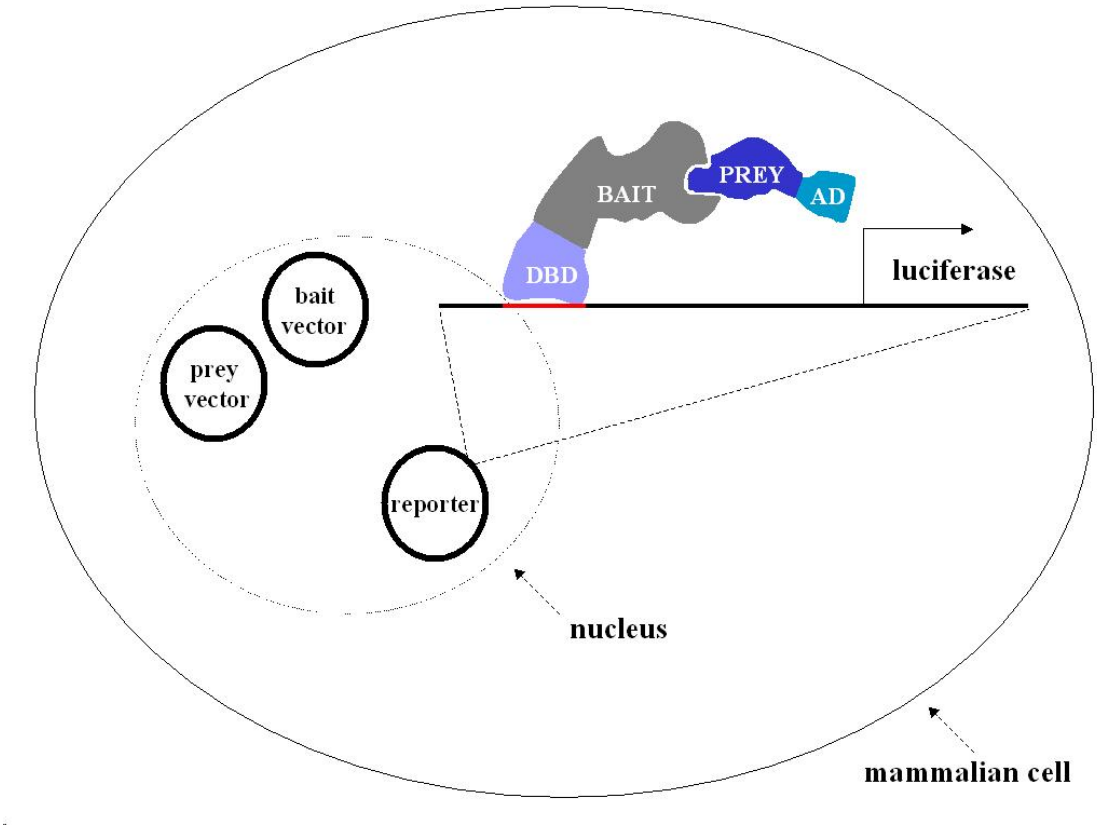

Figure 4 Schematic representation of the mammalian two-hybrid system
For explanation see text. The GAL4 binding site upstream of the luciferase marker is indicated in red.
Notes:
(1) The activation domain in the prey hybrid in the mammalian two-hybrid system is the AD of VP16, and not the AD of GAL4 as used in the yeast two-hybrid system.
(2) A mammalian cell is host for the protein-protein interaction. This cell cannot be seen a an empty tube in wich the interaction takes place. After synthesis of the hybrids in the cytoplasm, bait and prey may be modified by endogenous proteins (see also Chapter 4, Paragraph 4.9).

References

References are listed on pages 88-93.

References

References

Abu-Amer, Y. (2001). *J Clin Invest*, **107,** 1375-85.

Algul, H., Adler, G. & Schmid, R.M. (2002). *Int J Gastrointest Cancer*, **31,** 71-8.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. & Karin, M. (1987). *Cell*, **49,** 729-39.

Bakin, R.E., Gioeli, D., Bissonette, E.A. & Weber, M.J. (2003a). *Cancer Res*, **63,** 1975-80.

Bakin, R.E., Gioeli, D., Sikes, R.A., Bissonette, E.A. & Weber, M.J. (2003b). *Cancer Res*, **63,** 1981-9.

Barinaga, M. (1996). *Science*, **274,** 724.

Beg, A.A. & Baltimore, D. (1996). *Science*, **274,** 782-4.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N. & Dautry-Varsat, A. (1999). *J Cell Sci*, **112 (Pt 9),** 1303-11.

Bernard, D., Pourtier-Manzanedo, A., Gil, J. & Beach, D.H. (2003). *J Clin Invest*, **112,** 1724-31.

Birkenkamp, K.U., Geugien, M., Schepers, H., Westra, J., Lemmink, H.H. & Vellenga, E. (2004). *Leukemia*, **18,** 103-12.

Biswas, D.K., Cruz, A.P., Gansberger, E. & Pardee, A.B. (2000). *Proc Natl Acad Sci U S A*, **97,** 8542-7.

Bos, J.L. (1998). *Embo J*, **17,** 6776-82.

Bradley, J.R. & Pober, J.S. (2001). *Oncogene*, **20,** 6482-91.

Brinkmann, A.O. & Trapman, J. (2000). *Nat Med*, **6,** 628-9.

Buday, L. & Downward, J. (1993). *Cell*, **73,** 611-20.

Cantor, S.B., Urano, T. & Feig, L.A. (1995). *Mol Cell Biol*, **15,** 4578-84.

Cao, Y. & Karin, M. (2003). *J Mammary Gland Biol Neoplasia*, **8,** 215-23.

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. & Goeddel, D.V. (1996). *Nature*, **383,** 443-6.

Catz, S.D. & Johnson, J.L. (2001). *Oncogene*, **20,** 7342-51.

Ceresa, B.P., Kao, A.W., Santeler, S.R. & Pessin, J.E. (1998). *Mol Cell Biol*, **18,** 3862-70.

Chang, G.T., Blok, L.J., Steenbeek, M., Veldscholte, J., van Weerden, W.M., van Steenbrugge, G.J. & Brinkmann, A.O. (1997). *Cancer Res*, **57,** 4075-81.

Chen, C.D. & Sawyers, C.L. (2002). *Mol Cell Biol*, **22,** 2862-70.

Chen, F.E., Huang, D.B., Chen, Y.Q. & Ghosh, G. (1998a). *Nature*, **391,** 410-3.

Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P. & De Camilli, P. (1998b). *Nature*, **394,** 793-7.

Chen, L.F. & Greene, W.C. (2004). *Nat Rev Mol Cell Biol*, **5,** 392-401.

Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J.E., Jr. & Kuriyan, J. (1998c). *Cell*, **93,** 827-39.

Chiao, P.J., Miyamoto, S. & Verma, I.M. (1994). *Proc Natl Acad Sci U S A*, **91,** 28-32.

Colombel, M., Symmans, F., Gil, S., O'Toole, K.M., Chopin, D., Benson, M., Olsson, C.A., Korsmeyer, S. & Buttyan, R. (1993). *Am J Pathol*, **143,** 390-400.

Confalonieri, S. & Di Fiore, P.P. (2002). *FEBS Lett*, **513,** 24-9.

Coppe, J.P., Itahana, Y., Moore, D.H., Bennington, J.L. & Desprez, P.Y. (2004). *Clin Cancer Res*, **10,** 2044-51.

Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T. & Gutkind, J.S. (1995). *Cell*, **81,** 1137-46.
Craft, N., Shostak, Y., Carey, M. & Sawyers, C.L. (1999). *Nat Med*, **5,** 280-5.
Cukierman, E., Huber, I., Rotman, M. & Cassel, D. (1995). *Science*, **270,** 1999-2002.
Culig, Z., Hobisch, A., Cronauer, M.V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G. & Klocker, H. (1994). *Cancer Res*, **54,** 5474-8.
Davis, J.N., Kucuk, O. & Sarkar, F.H. (1999). *Nutr Cancer*, **35,** 167-74.
de Beer, T., Carter, R.E., Lobel-Rice, K.E., Sorkin, A. & Overduin, M. (1998). *Science*, **281,** 1357-60.
de Beer, T., Hoofnagle, A.N., Enmon, J.L., Bowers, R.C., Yamabhai, M., Kay, B.K. & Overduin, M. (2000). *Nat Struct Biol*, **7,** 1018-22.
Denmeade, S.R., Lin, X.S. & Isaacs, J.T. (1996). *Prostate*, **28,** 251-65.
Dhanalakshmi, S., Agarwal, R. & Agarwal, C. (2003). *Int J Oncol*, **23,** 721-7.
Di Fiore, P.P. & Gill, G.N. (1999). *Curr Opin Cell Biol*, **11,** 483-8.
Donaldson, J.G. & Jackson, C.L. (2000). *Curr Opin Cell Biol*, **12,** 475-82.
Egan, S.E., Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M. & Weinberg, R.A. (1993). *Nature*, **363,** 45-51.
Ellwood-Yen, K., Graeber, T.G., Wongvipat, J., Iruela-Arispe, M.L., Zhang, J., Matusik, R., Thomas, G.V. & Sawyers, C.L. (2003). *Cancer Cell*, **4,** 223-38.
Engedal, N., Korkmaz, C.G. & Saatcioglu, F. (2002). *Oncogene*, **21,** 1017-27.
Enmon, J.L., de Beer, T. & Overduin, M. (2000). *Biochemistry*, **39,** 4309-19.
Fazioli, F., Minichiello, L., Matoskova, B., Wong, W.T. & Di Fiore, P.P. (1993). *Mol Cell Biol*, **13,** 5814-28.
Feig, L.A., Urano, T. & Cantor, S. (1996). *Trends Biochem Sci*, **21,** 438-41.
Fields, S. & Song, O. (1989). *Nature*, **340,** 245-6.
Fleckenstein, D.S., Dirks, W.G., Drexler, H.G. & Quentmeier, H. (2003). *Leuk Res*, **27,** 687-94.
Floyd, S. & De Camilli, P. (1998). *Trends Cell Biol*, **8,** 299-301.
Ford, M.G., Mills, I.G., Peter, B.J., Vallis, Y., Praefcke, G.J., Evans, P.R. & McMahon, H.T. (2002). *Nature*, **419,** 361-6.
Fujii, T., Garcia-Bermejo, M.L., Bernabo, J.L., Caamano, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S.H. & Kazanietz, M.G. (2000). *J Biol Chem*, **275,** 7574-82.
Furuya, Y., Krajewski, S., Epstein, J.I., Reed, J.C. & Isaacs, J.T. (1996). *Clin Cancer Res*, **2,** 389-98.
Garg, A. & Aggarwal, B.B. (2002). *Leukemia*, **16,** 1053-68.
Gasparian, A.V., Yao, Y.J., Kowalczyk, D., Lyakh, L.A., Karseladze, A., Slaga, T.J. & Budunova, I.V. (2002). *J Cell Sci*, **115,** 141-51.
Ghosh, S., May, M.J. & Kopp, E.B. (1998). *Annu Rev Immunol*, **16,** 225-60.
Gietz, R.D. & Woods, R.A. (2002). *Methods Enzymol*, **350,** 87-96.
Gilmore, T.D. (1999). *Oncogene*, **18,** 6842-4.
Glauner, H., Siegmund, D., Motejadded, H., Scheurich, P., Henkler, F., Janssen, O. & Wajant, H. (2002). *Eur J Biochem*, **269,** 4819-29.
Gleave, M., Tolcher, A., Miyake, H., Nelson, C., Brown, B., Beraldi, E. & Goldie, J. (1999). *Clin Cancer Res*, **5,** 2891-8.
Gregory, C.W., He, B., Johnson, R.T., Ford, O.H., Mohler, J.L., French, F.S. & Wilson, E.M. (2001a). *Cancer Res*, **61,** 4315-9.
Gregory, C.W., Johnson, R.T., Jr., Mohler, J.L., French, F.S. & Wilson, E.M. (2001b). *Cancer Res*, **61,** 2892-8.
Hayden, M.S. & Ghosh, S. (2004). *Genes Dev*, **18,** 2195-224.
Heinlein, C.A. & Chang, C. (2002). *Endocr Rev*, **23,** 175-200.

Henry, D.O., Moskalenko, S.A., Kaur, K.J., Fu, M., Pestell, R.G., Camonis, J.H. & White, M.A. (2000). *Mol Cell Biol*, **20,** 8084-92.
Hoberg, J.E., Yeung, F. & Mayo, M.W. (2004). *Mol Cell*, **16,** 245-55.
Hoffman, C.S. & Winston, F. (1987). *Gene*, **57,** 267-72.
Horoszewicz, J.S., Leong, S.S., Chu, T.M., Wajsman, Z.L., Friedman, M., Papsidero, L., Kim, U., Chai, L.S., Kakati, S., Arya, S.K. & Sandberg, A.A. (1980). *Prog Clin Biol Res*, **37,** 115-32.
Huang, S., Pettaway, C.A., Uehara, H., Bucana, C.D. & Fidler, I.J. (2001). *Oncogene*, **20,** 4188-97.
Huggins, C. (1967). *Cancer Res*, **27,** 1925-30.
Huxford, T., Huang, D.B., Malek, S. & Ghosh, G. (1998). *Cell*, **95,** 759-70.
Ihle, J.N. & Kerr, I.M. (1995). *Trends Genet*, **11,** 69-74.
Ikeda, M., Ishida, O., Hinoi, T., Kishida, S. & Kikuchi, A. (1998). *J Biol Chem*, **273,** 814-21.
Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T. & Inoue, J. (1996). *J Biol Chem*, **271,** 28745-8.
Iversen, P.L., Arora, V., Acker, A.J., Mason, D.H. & Devi, G.R. (2003). *Clin Cancer Res*, **9,** 2510-9.
Jacobs, M.D. & Harrison, S.C. (1998). *Cell*, **95,** 749-58.
Janssen, T., Kiss, R., Dedecker, R., Petein, M., Pasteels, J.L. & Schulman, C. (1995). *Prostate*, **27,** 277-86.
Jefferies, C.A. & O'Neill, L.A. (2000). *J Biol Chem*, **275,** 3114-20.
Joneson, T. & Bar-Sagi, D. (1999). *Mol Cell Biol*, **19,** 5892-901.
Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gacon, G. & Camonis, J.H. (1995). *J Biol Chem*, **270,** 22473-7.
Jullien-Flores, V., Mahe, Y., Mirey, G., Leprince, C., Meunier-Bisceuil, B., Sorkin, A. & Camonis, J.H. (2000). *J Cell Sci*, **113,** 2837-44.
Karin, M. (1998). *Cancer J Sci Am*, **4 Suppl 1,** S92-9.
Karin, M. (1999). *Oncogene*, **18,** 6867-74.
Kariya, K., Koyama, S., Nakashima, S., Oshiro, T., Morinaka, K. & Kikuchi, A. (2000). *J Biol Chem*.
Kay, B.K., Williamson, M.P. & Sudol, M. (2000). *Faseb J*, **14,** 231-41.
Kimura, K. & Gelmann, E.P. (2002). *Cell Death Differ*, **9,** 972-80.
Koivisto, P., Kononen, J., Palmberg, C., Tammela, T., Hyytinen, E., Isola, J., Trapman, J., Cleutjens, K., Noordzij, A., Visakorpi, T. & Kallioniemi, O.P. (1997). *Cancer Res*, **57,** 314-9.
Kopp, E. & Ghosh, S. (1994). *Science*, **265,** 956-9.
Kozak, M. (1987). *Nucleic Acids Res*, **15,** 8125-48.
Levkau, B., Scatena, M., Giachelli, C.M., Ross, R. & Raines, E.W. (1999). *Nat Cell Biol*, **1,** 227-33.
Lindholm, P.F., Bub, J., Kaul, S., Shidham, V.B. & Kajdacsy-Balla, A. (2000). *Clin Exp Metastasis*, **18,** 471-9.
Ling, M.T., Wang, X., Lee, D.T., Tam, P.C., Tsao, S.W. & Wong, Y.C. (2004). *Carcinogenesis*, **25,** 517-25.
Ling, M.T., Wang, X., Ouyang, X.S., Xu, K., Tsao, S.W. & Wong, Y.C. (2003). *Oncogene*, **22,** 4498-508.
Liu, A.Y., Corey, E., Bladou, F., Lange, P.H. & Vessella, R.L. (1996). *Int J Cancer*, **65,** 85-9.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D. & Schlessinger, J. (1992). *Cell*, **70,** 431-42.

Lupas, A., Van Dyke, M. & Stock, J. (1991). *Science*, **252,** 1162-4.

Manna, S.K. & Aggarwal, B.B. (2000). *FEBS Lett*, **473,** 113-8.

Marchler-Bauer, A., Anderson, J.B., DeWeese-Scott, C., Fedorova, N.D., Geer, L.Y., He, S., Hurwitz, D.I., Jackson, J.D., Jacobs, A.R., Lanczycki, C.J., Liebert, C.A., Liu, C., Madej, T., Marchler, G.H., Mazumder, R., Nikolskaya, A.N., Panchenko, A.R., Rao, B.S., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Vasudevan, S., Wang, Y., Yamashita, R.A., Yin, J.J. & Bryant, S.H. (2003). *Nucleic Acids Res*, **31,** 383-7.

Masuda, A., Matsuguchi, T., Yamaki, K., Hayakawa, T. & Yoshikai, Y. (2001). *J Biol Chem*, **276,** 26107-13.

Masui, H., Wells, A., Lazar, C.S., Rosenfeld, M.G. & Gill, G.N. (1991). *Cancer Res*, **51,** 6170-5.

Masui, O., Ueda, Y., Tsumura, A., Koyanagi, M., Hijikata, M. & Shimotohno, K. (2002). *Int J Mol Med*, **9,** 489-93.

Matsubara, K., Hinoi, T., Koyama, S. & Kikuchi, A. (1997). *FEBS Lett*, **410,** 169-74.

Mayo, M.W., Wang, C.Y., Cogswell, P.C., Rogers-Graham, K.S., Lowe, S.W., Der, C.J. & Baldwin, A.S., Jr. (1997). *Science*, **278,** 1812-5.

McDonnell, T.J., Troncoso, P., Brisbay, S.M., Logothetis, C., Chung, L.W., Hsieh, J.T., Tu, S.M. & Campbell, M.L. (1992). *Cancer Res*, **52,** 6940-4.

McKenna, N.J., Lanz, R.B. & O'Malley, B.W. (1999). *Endocr Rev*, **20,** 321-44.

Mercurio, F. & Manning, A.M. (1999). *Oncogene*, **18,** 6163-71.

Moriggl, R., Berchtold, S., Friedrich, K., Standke, G.J., Kammer, W., Heim, M., Wissler, M., Stocklin, E., Gouilleux, F. & Groner, B. (1997). *Mol Cell Biol*, **17,** 3663-78.

Morinaka, K., Koyama, S., Nakashima, S., Hinoi, T., Okawa, K., Iwamatsu, A. & Kikuchi, A. (1999). *Oncogene*, **18,** 5915-22.

Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C.F., Yagita, H. & Okumura, K. (1996). *J Biol Chem*, **271,** 14661-4.

Nakashima, S., Morinaka, K., Koyama, S., Ikeda, M., Kishida, M., Okawa, K., Iwamatsu, A., Kishida, S. & Kikuchi, A. (1999). *Embo J*, **18,** 3629-42.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D.A., Goulet, R.J., Jr. & Sledge, G.W., Jr. (1997). *Mol Cell Biol*, **17,** 3629-39.

Nelson, G., Wilde, G.J., Spiller, D.G., Kennedy, S.M., Ray, D.W., Sullivan, E., Unitt, J.F. & White, M.R. (2003). *J Cell Sci*, **116,** 2495-503.

Ni, Z., Lou, W., Lee, S.O., Dhir, R., DeMiguel, F., Grandis, J.R. & Gao, A.C. (2002). *J Urol*, **167,** 1859-62.

Norris, J.L. & Baldwin, A.S., Jr. (1999). *J Biol Chem*, **274,** 13841-6.

Olivier, J.P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993). *Cell*, **73,** 179-91.

Oosterhoff, J.K., Penninkhof, F., Brinkmann, A.O., Anton Grootegoed, J. & Blok, L.J. (2003). *Oncogene*, **22,** 2920-5.

Orlowski, R.Z. & Baldwin, A.S. (2002). *Trends Mol Med*, **8,** 385-9.

Osada, M., Tolkacheva, T., Li, W., Chan, T.O., Tsichlis, P.N., Saez, R., Kimmelman, A.C. & Chan, A.M. (1999). *Mol Cell Biol*, **19,** 6333-44.

Oshiro, T., Koyama, S., Sugiyama, S., Kondo, A., Onodera, Y., Asahara, T., Sabe, H. & Kikuchi, A. (2002). *J Biol Chem*, **277,** 38618-26.

Ouyang, X.S., Wang, X., Lee, D.T., Tsao, S.W. & Wong, Y.C. (2002). *J Urol*, **167,** 2598-602.

Pahl, H.L. (1999). *Oncogene*, **18,** 6853-66.
Palayoor, S.T., Youmell, M.Y., Calderwood, S.K., Coleman, C.N. & Price, B.D. (1999). *Oncogene*, **18,** 7389-94.
Panayotou, G. & Waterfield, M.D. (1993). *Bioessays*, **15,** 171-7.
Paoluzi, S., Castagnoli, L., Lauro, I., Salcini, A.E., Coda, L., Fre, S., Confalonieri, S., Pelicci, P.G., Di Fiore, P.P. & Cesareni, G. (1998). *Embo J*, **17,** 6541-50.
Park, J.I., Lee, M.G., Cho, K., Park, B.J., Chae, K.S., Byun, D.S., Ryu, B.K., Park, Y.K. & Chi, S.G. (2003). *Oncogene*, **22,** 4314-32.
Park, S.H. & Weinberg, R.A. (1995). *Oncogene*, **11,** 2349-55.
Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R. & Lacal, J.C. (1997). *Genes Dev*, **11,** 463-75.
Pruitt, K. & Der, C.J. (2001). *Cancer Letters*, **171,** 1-10.
Randazzo, P.A. & Hirsch, D.S. (2004). *Cell Signal*, **16,** 401-13.
Randazzo, P.A., Nie, Z., Miura, K. & Hsu, V.W. (2000). *Sci STKE*, **2000,** RE1.
Rosse, C., L'Hoste, S., Offner, N., Picard, A. & Camonis, J. (2003). *J Biol Chem*, **278,** 30597-604.
Rothe, M., Sarma, V., Dixit, V.M. & Goeddel, D.V. (1995). *Science*, **269,** 1424-7.
Russell, P.J., Bennett, S. & Stricker, P. (1998). *Clin Chem*, **44,** 705-23.
Salcini, A.E., Chen, H., Iannolo, G., De Camilli, P. & Di Fiore, P.P. (1999). *Int J Biochem Cell Biol*, **31,** 805-9.
Salcini, A.E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P.G. & Di Fiore, P.P. (1997). *Genes Dev*, **11,** 2239-49.
Santolini, E., Salcini, A.E., Kay, B.K., Yamabhai, M. & Di Fiore, P.P. (1999). *Exp Cell Res*, **253,** 186-209.
Schroder, F.H., Hermanek, P., Denis, L., Fair, W.R., Gospodarowicz, M.K. & Pavone-Macaluso, M. (1992). *Prostate Suppl*, **4,** 129-38.
Sclabas, G.M., Fujioka, S., Schmidt, C., Evans, D.B. & Chiao, P.J. (2003). *Int J Gastrointest Cancer*, **33,** 15-26.
Sen, R. & Baltimore, D. (1986). *Cell*, **47,** 921-8.
Sensibar, J.A., Sutkowski, D.M., Raffo, A., Buttyan, R., Griswold, M.D., Sylvester, S.R., Kozlowski, J.M. & Lee, C. (1995). *Cancer Res*, **55,** 2431-7.
Shen, C.H. & Stavnezer, J. (1998). *Mol Cell Biol*, **18,** 3395-404.
Shields, J.M., Pruitt, K., McFall, A., Shaub, A. & Der, C.J. (2000). *Trends Cell Biol*, **10,** 147-54.
Shukla, S. & Gupta, S. (2004). *Clin Cancer Res*, **10,** 3169-78.
Simon, M.A., Dodson, G.S. & Rubin, G.M. (1993). *Cell*, **73,** 169-77.
Sorkin, A. & Waters, C.M. (1993). *Bioessays*, **15,** 375-82.
Steiner, H., Godoy-Tundidor, S., Rogatsch, H., Berger, A.P., Fuchs, D., Comuzzi, B., Bartsch, G., Hobisch, A. & Culig, Z. (2003). *Am J Pathol*, **162,** 655-63.
Steketee, K., Timmerman, L., Ziel-van der Made, A.C., Doesburg, P., Brinkmann, A.O. & Trapman, J. (2002). *Int J Cancer*, **100,** 309-17.
Sumitomo, M., Tachibana, M., Nakashima, J., Murai, M., Miyajima, A., Kimura, F., Hayakawa, M. & Nakamura, H. (1999). *J Urol*, **161,** 674-9.
Takeda, K., Kishimoto, T. & Akira, S. (1997). *J Mol Med*, **75,** 317-26.
Turner, C.E., West, K.A. & Brown, M.C. (2001). *Curr Opin Cell Biol*, **13,** 593-9.
Ui, M., Okada, T., Hazeki, K. & Hazeki, O. (1995). *Trends Biochem Sci*, **20,** 303-7.
Uzzo, R.G., Leavis, P., Hatch, W., Gabai, V.L., Dulin, N., Zvartau, N. & Kolenko, V.M. (2002). *Clin Cancer Res*, **8,** 3579-83.
Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. & Verma, I.M. (1996). *Science*, **274,** 787-9.

van Bokhoven, A., Varella-Garcia, M., Korch, C. & Miller, G.J. (2001). *Cancer Res*, **61,** 6340-4.

van Steenbrugge, G.J., van Uffelen, C.J., Bolt, J. & Schroder, F.H. (1991). *J Steroid Biochem Mol Biol*, **40,** 207-14.

van Weerden, W.M., de Ridder, C.M., Verdaasdonk, C.L., Romijn, J.C., van der Kwast, T.H., Schroder, F.H. & van Steenbrugge, G.J. (1996). *Am J Pathol*, **149,** 1055-62.

Veldscholte, J., Ris-Stalpers, C., Kuiper, G.G., Jenster, G., Berrevoets, C., Claassen, E., van Rooij, H.C., Trapman, J., Brinkmann, A.O. & Mulder, E. (1990). *Biochem Biophys Res Commun*, **173,** 534-40.

Vertegaal, A.C., Kuiperij, H.B., Yamaoka, S., Courtois, G., van der Eb, A.J. & Zantema, A. (2000). *Cell Signal*, **12,** 759-68.

Vieira, A.V., Lamaze, C. & Schmid, S.L. (1996). *Science*, **274,** 2086-9.

Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J. & Kallioniemi, O.P. (1995). *Nat Genet*, **9,** 401-6.

Wajant, H., Henkler, F. & Scheurich, P. (2001). *Cell Signal*, **13,** 389-400.

Wang, C.Y., Mayo, M.W. & Baldwin, A.S., Jr. (1996). *Science*, **274,** 784-7.

Ware, J.L. (1998). *Cancer Metastasis Rev*, **17,** 443-7.

Wells, A., Welsh, J.B., Lazar, C.S., Wiley, H.S., Gill, G.N. & Rosenfeld, M.G. (1990). *Science*, **247,** 962-4.

Wolthuis, R.M., Zwartkruis, F., Moen, T.C. & Bos, J.L. (1998). *Curr Biol*, **8,** 471-4.

Wong, W.T., Schumacher, C., Salcini, A.E., Romano, A., Castagnino, P., Pelicci, P.G. & Di Fiore, P. (1995). *Proc Natl Acad Sci U S A*, **92,** 9530-4.

Ye, X., Mehlen, P., Rabizadeh, S., VanArsdale, T., Zhang, H., Shin, H., Wang, J.J., Leo, E., Zapata, J., Hauser, C.A., Reed, J.C. & Bredesen, D.E. (1999). *J Biol Chem*, **274,** 30202-8.

Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A. & Mayo, M.W. (2004). *Embo J*, **23,** 2369-80.

Yin, M.J., Yamamoto, Y. & Gaynor, R.B. (1998). *Nature*, **396,** 77-80.

Zerbini, L.F., Wang, Y., Cho, J.Y. & Libermann, T.A. (2003). *Cancer Res*, **63,** 2206-15.

Zhao, X.Y., Malloy, P.J., Krishnan, A.V., Swami, S., Navone, N.M., Peehl, D.M. & Feldman, D. (2000). *Nat Med*, **6,** 703-6.

Summary

Summary

Prostate cancer can be treated by androgen ablation therapy, because prostate cancer cells are dependent on androgens for growth and survival. However, after a period of time some of the prostate cancer cells no longer respond to androgen ablation and survive the therapy. This transition of androgen-dependent prostate cancer (ADPC) to androgen-independent prostate cancer (AIPC) is a serious health problem, since no effective therapy is available for this androgen-independent stage of the disease. The molecular mechanisms that underlie the transition are largely unknown. REPS2, which is the protein that is studied in this thesis, might be involved in a molecular mechanism that contributes to AIPC development, since REPS2 mRNA is downregulated in AIPC compared to ADPC.

The main question addressed in this thesis is:

How can a decrease in REPS2 expression contribute to survival of prostate cancer cells?

Antibodies were raised against peptides representing regions of REPS2. Using these antibodies it was shown that the REPS2 protein level in AIPC is decreased compared to ADPC (Chapter 2). Transient overexpression of REPS2 in prostate cancer cell lines induced apoptosis within 48 h (Chapter 2), which indicates that REPS2 may play a role in the life-death balance of the cell.
Roles downstream of the epidermal growth factor (EGF) receptor and in receptor mediated endocytosis (RME) have been described for REPS2. Therefore it was hypothesized that downregulation of REPS2 in AIPC leads to disturbed RME and decreased internalisation of the EGF receptor, which may cause a continuous growth signal. This might be a powerful mechanism by which prostate cancer cells can survive and grow at low levels of androgens.
To elucidate cellular functions of REPS2, proteins were identified that bind REPS2. Therefore REPS2 was used as bait in a yeast two-hybrid screen. In this screen several human protein sequences bound physically to REPS2 in yeast cells (Appendix).
Chapter 3 describes interaction between REPS2 and a large fragment of the NF-κB subunit p65 (RELA). p65 is involved in cell survival and possibly plays an important role in prostate cancer progression, because p65 is inactive in ADPC but active in AIPC. Therefore, the interaction between REPS2 and p65 was studied further. With additional protein-protein interaction assays in yeast it was found that the EPS15 homology (EH) domain of REPS2 bound to the NPF region of p65. The observed interaction is in good agreement with published data, because EH domains have been described as protein-protein interaction domains that recognize proteins containing NPF sequences. Crystal structure data from literature and experimental data obtained with a mutated REPS2 EH domain provided additional support for the observed interaction. To confirm the observed interaction in mammalian cells, REPS2-p65 mammalian protein interaction assays were conducted in COS-1 cells and LNCaP cells (derived from human prostate cancer lymph node metastasis). Not all the bindings between full length or various truncated forms of REPS2 and p65 that were detected in yeast cells could be observed also in mammalian cells. From this, we suggested that the REPS2-p65 interaction in mammalian cells might be regulated, and might be stimulated by activation of intracellular signal transduction pathways. We

used PMA (phorbol ester) to affect intracellular regulation mechanisms that possibly are important for REPS2-p65 interaction. PMA activates intracellular signalling pathways, including pathways that lead to NF-κB activation, and the results indicate that this signalling exerts a positive effect on REPS2-p65 binding in LNCaP cells.

In Chapter 4 the effect of REPS2 expression on a constitutively active NF-κB pathway in TSU cells (human bladder cancer cells) is discussed. This Chapter also shows extended data on the REPS2-p65 interaction. Preliminary results indicate that intracellular signalling pathways induced by TNFα stimulate REPS2-p65 binding in LNCaP cells. Chapter 4 furthermore describes interactions between REPS2 and other protein sequences that were identified in the yeast two-hybrid screen. Two of the identified protein sequences represent parts of TRAF4 (TNF receptor-associated factor 4) and STAT6 (signal transducer and activator of transcription 6). Interestingly, these two proteins, like p65, are involved in cell survival and implicated in some aspects of the NF-κB pathway. With the use of additional yeast two-hybrid assays, the REPS2-TRAF4 and REPS2-STAT6 binding regions were determined. Preliminary data showed that the coiled-coil region of REPS2 might bind the coiled-coil region in TRAF4, whereas the EH domain of REPS2 might bind the FW sequence of STAT6 (FW sequences are a second class of motifs that can bind EH domains). Furthermore, it is demonstrated that PMA-activated signalling stimulates REPS2-TRAF4 binding in LNCaP cells.

The hypothesis could not be supported, since the present identification and characterization of novel REPS2 binding partners, do not provide information that directly relates to the putative role of REPS2 in receptor-mediated endocytosis. However, the results point to a putative role of REPS2 in NF-κB signalling and control of cell survival. This would be in agreement with the observed apoptosis-inducing effect of REPS2 over-expression in prostate cancer cells. Through a direct involvement in protein-protein complexes and interactions, REPS2 might provide a connection between EGF signalling and NF-κB signalling. It is to be expected that the decreased expression level of REPS2 protein in AIPC will lead to partial loss of protein-protein interactions in which REPS2 is involved, and this may evoke changes in the cellular life-death balance, allowing the cells to survive and grow also when the cells are exposed to a low level of androgenic stimulation. Implications for our understanding of mechanisms that control progression of prostate cancer, are discussed in Chapter 5.

Samenvatting

Samenvatting

Prostaatkanker kan met androgeen onttrekkings-therapie worden behandeld, omdat prostaatkankercellen afhankelijk zijn van androgenen voor groei en overleving. Echter, na een bepaalde periode reageren sommige prostaatkankercellen niet meer op de onttrekking van androgenen. Deze overgang van androgeen-afhankelijke prostaatkanker naar androgeen-onafhankelijke prostaatkanker is een ernstig probleem, omdat voor androgeen-onafhankelijke prostaatkanker geen effectieve therapie beschikbaar is. De moleculaire mechanismen die aan de overgang naar androgeen-onafhankelijke groei ten grondslag liggen zijn grotendeels onbekend. REPS2, het eiwit dat werd bestudeerd in het onderzoek beschreven in dit proefschrift, zou direct of indirect betrokken kunnen zijn in een moleculair mechanisme dat bijdraagt aan de ontwikkeling van androgeen-onafhankelijke prostaatkanker. Het REPS2 mRNA niveau is in androgeen-afhankelijke prostaatkanker veel hoger dan in androgeen-onafhankelijke prostaatkanker, en een rol van REPS2 zou verband kunnen houden met verlies van REPS2 expressie tijdens progressie naar androgeen-onafhankelijkheid.

De belangrijkste vraag die in dit proefschrift aan de orde wordt gesteld is:

Hoe kan een afname in REPS2 expressie bijdragen aan androgeen-onafhankelijke overleving en groei van prostaatkankercellen?

Antilichamen werden opgewekt tegen peptides die overeenkomen met aminozuur-sequenties uit REPS2. Met behulp van deze antilichamen kon worden aangetoond dat het REPS2 eiwitniveau in androgeen-onafhankelijke prostaatkanker is afgenomen, vergeleken met androgeen-afhankelijke prostaatkanker (Hoofdstuk 2). Tijdelijke overexpressie van REPS2 in prostaatkankercellen induceerde geprogrammeerde celdood, apoptose, binnen 48 uur (Hoofdstuk 2), wat aantoont dat REPS2 mogelijk een rol speelt in de “leven of dood”-balans binnen deze kankercellen.
Functies met betrekking tot regulatie van EGF signaaltransductie en endocytose van de EGF receptor zijn beschreven voor REPS2. Daarom werd de volgende hypothese geformuleerd: de afname van REPS2 in androgeen-onafhankelijke prostaatkanker leidt tot een verstoorde en verlaagde endocytose van de EGF receptor, wat mogelijk een continu groeisignaal tot gevolg heeft. Dit zou een mechanisme kunnen zijn dat bijdraagt aan de overleving van prostaatkankercellen bij een laag niveau van androgenen.
Om cellulaire functies van REPS2 in kaart te brengen werden enkele eiwitten geidentificeerd die aan REPS2 kunnen binden. Om deze identificatie te kunnen uitvoeren werd REPS2 als lokaas gebruikt in een zogenaamde ‘yeast two-hybrid screen’. In deze screen bonden verschillende humane eiwitten aan REPS2 in gistcellen (Appendix).
Hoofdstuk 3 omschrijft de interactie tussen REPS2 en een groot fragment van p65, een van de eiwitten van het NF-kappaB complex. NF-kappaB kan een rol spelen in verschillende cellulaire processen, en het stimuleren van cellulaire overlevingsmechanismen is een van die processen. Het p65 eiwit speelt mogelijk een belangrijke rol in de progressie van prostaatkanker, omdat p65 inactief is in androgeen-afhankelijke prostaatkanker maar actief is in androgeen-onafhankelijke

prostaatkanker. Om die reden werd de interactie tussen REPS2 en p65 in meer detail bestudeerd.
In additionele eiwit-eiwit interactie experimenten in gist werd aangetoond dat het EPS15 homologie (EH) domein van REPS2 aan de NPF regio van p65 bindt. Deze gevonden interactie is in goede overeenstemming met gepubliceerde data, omdat EH domeinen worden omschreven als eiwit-eiwit interactie domeinen die binden aan eiwitten met NPF sequenties. Kristalstructuur data uit de literatuur en experimentele data verkregen met een gemuteerd REPS2 EH domein gaven extra ondersteuning voor de waargenomen interactie. Om deze interactie in zoogdiercellen te bevestigen, werden REPS2-p65 eiwit-eiwit interactie experimenten in COS1 (een apen nier-cellijn) en LNCaP (humane prostaatkanker-cellijn) uitgevoerd. Niet alle bindingen tussen complete of verkorte vormen van REPS2 en p65 die in gist waren gedetecteerd konden ook worden aangetoond in zoogdiercellen. Gebaseerd op deze waarnemingen suggereerden wij dat REPS2-p65 binding in zoogdiercellen mogelijk wordt gereguleerd, en mogelijk gestimuleerd kan worden door activatie van intracellulaire signaaltransductie paden. PMA (phorbol ester) werd gebruikt om intracellulaire regulatiemechanismen te beinvloeden, die mogelijk belangrijk zijn voor REPS2-p65 interactie. PMA activeert intracellulaire signaaltransductie-paden, waaronder paden die leiden tot activatie van NF-kappaB, en de resultaten laten zien dat PMA-geinduceerde signaaltransductie een positief effect heeft op REPS2-p65 binding in LNCaP cellen.
In Hoofdstuk 4 worden waargenomen effecten van REPS2 expressie op een constitutief actief NF-kappaB in TSU cellen (humane blaaskankercellen) omschreven. Tevens bevat dit hoofdstuk extra data over de REPS2-p65 interactie. Voorlopige resultaten geven aan dat TNFα-geinduceerde signaaltransductie-paden REPS2-p65 interactie kunnen stimuleren in LNCaP cellen. Hoofdstuk 4 omschrijft ook de interactie tussen REPS2 en andere aminozuur-sequenties die in de yeast two-hybrid screen geidentificeerd zijn. Twee van deze sequenties representeren delen van TRAF4 (TNF receptor-associated factor 4) en STAT6 (signal transducer and activator of transcription 6). Het is interessant dat deze twee eiwitten, net als p65, betrokken zijn bij cellulaire overlevingsmechanismen en een rol spelen in sommige aspecten van NF-kappaB signaaltransductie. Met gebruikmaking van doelgerichte eiwit-eiwit interactie experimenten in gist werd onderzocht welke aminozuursequenties betrokken zijn bij de REPS2-TRAF4 en REPS2-STAT6 binding. Preliminaire data laat zien dat een coiled-coil domein van REPS2 kan binden aan een coiled-coil domein van TRAF4, terwijl het EH domein in REPS2 mogelijkerwijs de FW sequentie in STAT6 bindt (FW sequenties zijn een tweede klasse van motieven die aan EH domeinen kunnen binden). Verder werd nog getoond dat PMA-geactiveerde signaaltransductie REPS2-TRAF4 binding kan stimuleren in LNCaP cellen.
De huidige identificatie en karakterisatie van nog niet eerder aangetoonde REPS2 eiwitpartners geeft geen informatie die direct gekoppeld kan worden aan een rol voor REPS2 in endocytose van receptoren. Echter, de resultaten wijzen naar een vermoedelijke rol voor REPS2 in NF-kappaB signaaltransductie en regulatie van cellulaire overlevingsmechanismen. Dit zou in overeenstemming zijn met de waargenomen inductie van apoptose door REPS2 over-expressie in prostaatkankercellen. Via een directe betrokkenheid in eiwit-eiwit complexen en interacties, zou REPS2 een koppeling kunnen bewerkstelligen tussen EGF en NF-kappaB signaal transductie. Het is te verwachten dat een daling van het REPS2 eiwitniveau in androgeen-onafhankelijke prostaatkanker zal leiden tot een gedeeltelijke afname van enkele eiwit-eiwit interacties waarbij REPS2 direct

betrokken is. Dit kan mogelijk veranderingen in de cellulaire “leven of dood”-balans teweegbrengen, en hierdoor cellen activeren om te overleven en te groeien onder omstandigheden van androgeen-onttrekking. Implicaties van de resultaten voor kennis over mechanismen betrokken bij progressie van prostaatkanker worden in Hoofstuk 5 besproken.

Dankwoord

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List of publications

Oosterhoff, J.K., Penninkhof, F., Brinkmann, A.O., Grootegoed, J.A., and Blok, L.J.
REPS2/POB1 is downregulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells.
Oncogene. 2003 May 15;22(19):2920-5.
http://www.nature.com/onc/journal/v22/n19/abs/1206397a.html

Penninkhof, F., Grootegoed, J.A., and Blok, L.J.
Identification of REPS2 as a putative modulator of NF-kappaB activity in prostate cancer cells.
Oncogene. 2004 July 22;23(33):5607-15.
http://www.nature.com/onc/journal/v23/n33/abs/1207750a.html

Curriculum vitae

Na de middelbare school (MAVO en VWO), het volgen van een 1 jarige informatica opleiding, het vervullen van de militaire diensplicht, en het uitoefenen van verschillende tijdelijke banen, begon de schrijver van dit proefschrift in 1992, toen net 23, aan zijn studie biologie in Groningen. Hij specialiseerde zich in de moleculaire biologie, en onderzocht tijdens zijn eerste afstudeerproject late genexpressie van de bacteriofaag r1t bij prof. dr. Gerard Venema (Vakgroep Moleculaire Genetica, Afdeling Biologie, Rijksuniversiteit Groningen). Bij diezelfde vakgroep kon, op eigen initiatief en naar eigen onderzoeksvoorstel, het tweede afstudeeronderwerp worden uitgevoerd. Dit onderwerp betrof functionele analyse van de complete genomen van de bacteriofaag r1t en de bacterie *Bacillus subtilis* met behulp van bioinformatica. Na afstuderen (in 1998) is de schrijver van dit proefschrift onder andere werkzaam geweest als adviseur omtrent millennium problematiek. In 1999 verhuisde hij naar Rotterdam om aan het in dit proefschrift beschreven onderzoek te beginnen bij prof. dr. Anton Grootegoed (Afdeling Voortplanting en Ontwikkeling, Erasmus MC, Universitair Medisch Centrum Rotterdam).